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TESIS DOCTORAL

IDENTIFICATION OF NEW KINASE SUBSTRATES INVOLVED IN THE DNA DAMAGE RESPONSE PATHWAY AND ITS IMPLICATION IN CARCINOGENESIS

Memoria presentada para optar al grado de Doctora en Biomedicina por

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TITULO: *Identification of new kinase substrates involved in the DNA Damage Response pathway and its implication in carcinogenesis*

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TÍTULO DE LA TESIS: Identification of new kinase substrates involved in the DNA Damage Response pathway and its implication in carcinogenesis.

DOCTORANDO/A: María Isabel Lara Chica

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

El documento presentado por la doctoranda María Isabel Lara Chica, con título "Identification of new kinase substrates involved in the DNA Damage Response pathway and its implication in carcinogenesis", corresponde a su trabajo de tesis doctoral realizado en el periodo comprendido entre Enero de 2013 y Junio de 2017. En este trabajo se han cumplido los objetivos establecidos al comienzo del proyecto, haciendo posible el aprendizaje de técnicas experimentales de gran relevancia y utilidad en el campo de la investigación de la biología molecular. Este trabajo ha permitido la obtención de resultados acerca de la funcionalidad de dos quinasas relevantes en la ruta de respuesta al daño al ADN, CHK2 y DYRK2, a través de la identificación de nuevos sustratos de las mismas. En concreto, se ha identificado a SIAH2 como sustrato de CHK2, existiendo una regulación muta entre ambas proteínas con influencia sobre la progresión del ciclo celular y la habilidad de la hipoxia para alterar la respuesta al daño al ADN en células cancerígenas. Además, en este trabajo se ha descrito como DYRK2 regula negativamente la expresión de CDC25A tanto en condiciones normales como en presencia de daño al ADN, entre otros estímulos, existiendo una correlación inversa de ambas proteínas en células y tejidos cáncer de pulmón. La finalidad del estudio ha sido compensada con la publicación de un artículo científico en una revista indexada dentro del primer cuartil de su área. De manera conjunta a la elaboración de este proyecto, se han realizado trabajos en apoyo a otros

miembros del grupo los cuales también han contribuido al avance y finalización de este trabajo dando lugar a otras publicaciones. Además, se ha dado difusión a los resultados obtenidos de todos los trabajos mediante la presentación de los mismos a jornadas y congresos siendo expuestos a través mediante comunicaciones orales.

PUBLICACIONES Y TRABAJOS DERIVADOS DE LA TESIS DOCTORAL

_ Publicaciones derivadas de la tesis doctoral.

García-Limones, C.*, **Lara-Chica, M.*,** Jiménez-Jiménez, C., Perez, M., Moreno, P., Muñoz, E., Calzado, M.A. **CHK2 stability is regulated by the E3 ubiquitin ligase SIAH2.** ONCOGENE. 2016, 35 - 33, pp. 4289 - 4301. IF: 7.932. Journal position: 15/213 (Q1). ***First co-authors.***

_ Publicaciones en colaboración durante el desarrollo de la tesis doctoral.

García, Víctor; **Lara-Chica, Maribel**; Cantarero, Irene; Sterner, Olov; Calzado, Marco A.; Muñoz, Eduardo. **Galiellalactone induces cell cycle arrest and apoptosis through the ATM/ATR pathway in prostate cancer cells.** ONCOTARGET. 2016, 7 - 4, pp. 4490 - 4506. IF: 5.008. Journal position: 36/213 (Q1).

Paula Moreno, **Maribel Lara-Chica,** Rafael Soler, Teresa Caro, Manuel Medina, Antonio Álvarez, Ángel Salvatierra, Eduardo Muñoz y Marco A. Calzado. **The Expression of the Ubiquitin Ligase SIAH2 (seven *in absentia* homolog 2) is Increased in Human Lung Cancer.** PLoS ONE. 2015, 18;10(11):e0143376. IF: 3,234. Journal position: 8/56 (Q1).

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_ **Comunicaciones presentadas a congresos internacionales.**

Maribel Lara-Chica; Rosario Morrugares Carmona; Carla Jiménez-Jiménez; Eduardo Muñoz Blanco; Marco A. Calzado Canale. **"Identification of new DYRK2 substrates and their implications in carcinogenesis".** DYRK1A, related kinases and human disease International Conference (ManROS Therapeutics), Saint Malo, Bretagne, France, March 28 – April 1st, 2017.

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Maribel Lara-Chica, Carmen García-Limones, Carla Jiménez-Jiménez, Moisés Pérez, Rosario Morrugares, Eduardo Muñoz, Marco A. Calzado. **"Key proteins regulation of the DNA Damage Response Pathway CHK2 and CDC25A in the context of carcinogenesis".** VII Young Researchers' Meeting, Córdoba, Spain, May 10-11th, 2016.

Maribel Lara-Chica, Carla Jiménez-Jiménez, Paula Moreno, Moisés Pérez, Eduardo Muñoz, Marco A. Calzado. **"Identification of new substrates for DYRK2 and its implication in carcinogenesis: CDC25A regulation in the context of lung cancer".** VI Young Researchers' Meeting, Córdoba, Spain, May 4-5th, 2015.

_ **Premios obtenidos.**

Premio a la mejor presentación oral en la sesión de Cáncer.

Maribel Lara-Chica, Carmen García-Limones, Carla Jiménez-Jiménez, Moisés Pérez, Rosario Morrugares, Eduardo Muñoz, Marco A. Calzado. **"Key proteins regulation of the DNA Damage Response Pathway CHK2 and CDC25A in the context of carcinogenesis".** VII Young Researchers' Meeting, Córdoba, Spain, May 10-11th, 2016.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 20 de Junio de 2017
Firma del/de los director/es

Fdo.: Marco A. Calzado Canale

Fdo.: Eduardo Muñoz Blanco

To my forever beloved Diego Chica García

“It is not the critic who counts,
Not the man who points out how the strong man stumbles,
Or where the doer of deeds could have done them better.
The credit belongs to the man that is actually in the arena”

THEODORE ROOSVELT

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Abstract

One of the most relevant altered pathways in cancer is the DNA Damage Response (DDR), a well-coordinated network of signalling cascades that cells have developed during evolution in order to maintain genomic integrity. These pathways are activated when genomic material is damaged with the aim of transmit the damage signals to effector proteins, and induce cellular responses including cell cycle arrest, activation of DNA repair pathways, and cell death; thus preventing tumorigenesis. CHK2 and DYRK2 are key downstream kinases of the DDR, involved mainly in the regulation of cell cycle and DNA repair. The identification of new substrates for these kinases will delve into knowledge of their functions within the DDR, which leads to tumor development and progression when deregulated.

In the present work we demonstrate how CHK2 presents the ability to phosphorylate the E3 Ubiquitin ligase SIAH2, affecting its activity on certain substrates. Additionally, SIAH2 regulates CHK2 basal turnover through ubiquitination and proteasomal degradation. In response to DNA damage, the interaction between both proteins is disrupted, which favors CHK2 stabilization. This new CHK2 regulation mechanism has an influence on cell cycle progression and on the ability of hypoxia to alter DDR pathway in cancer cells, since resistance to apoptosis induced by genotoxic agents in cells subjected to hypoxia could be partly explained by the mutual regulation between CHK2 and SIAH2. We also describe the capacity of DYRK2 to modulate the turnover of the checkpoint phosphatase CDC25A through an ubiquitin/proteasome and DYRK2 kinase activity dependent process. DYRK2 directly phosphorylates CDC25A *in vitro*, although DSG or KEN motifs are not involved in the CDC25A degradation by the proteasome mediated by DYRK2. Different stimuli, such as DNA damage or serum starvation, result in an inverse correlation of DYRK2 and CDC25A expression, which is also observed at endogenous levels in human lung cancer cell lines and human lung cancer tissues.

Taken together, our findings report new substrates (SIAH2 and CDC25A) for two relevant kinases involved in the DDR pathway (CHK2 and DYRK2), helping to elucidate the molecular mechanisms through which this pathway operates. These data certainly help to improve the understanding of the regulation processes of these relevant kinases and will extend the knowledge about the molecular biology of cancer as well as provide new therapeutic opportunities for cancer treatment.

Abbreviations

5-FU 5-Fluorouracil

ADR Adryamicine

ATM Ataxia telangiectasia mutated

ATR Ataxia telangiectasia and Rad3 related

BER Base excision repair

CDC25A Cell division cycle 25A

CHK2 Checkpoint kinase 2

CHX Cicloheximide

CPR Cisplatin resistant

DDR DNA Damage Response

DNA Desoxirribonucleic acid

DSB Double-strand break

DYRK2 Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2

ETP Etoposide

HIPK2 Homeodomain-interacting protein kinase 2

HR Homologous recombination

IP Immunoprecipitation

IR ionizing radiation

MMR Mismatch repair

NER Nucleotide excision repair

NHEJ Non-homologous end joining

Ni-NTA Nickel-nitrilotriacetic acid

NLS Nuclear localization signal

PHD Prolyl hydroxylase

qPCR Quantitative real time polymerase chain reaction

RING Really Interesting New Gene

RNA Ribonucleic acid

ROS reactive oxygen species

SIAH2 Seven in absentia homolog 2

SSB Single-strand breaks

UV Ultraviolet light

Introduction

3.1. DNA Damage Response

The search for new therapeutic targets and strategies is crucial in cancer research, not only for the development of new drugs, but also for use as prognostic markers which have become an important tool in cancer treatment. To achieve this aim, key molecular cell components that could be altered in carcinogenesis had been studied deeply over the last decade.

One of the most relevant altered pathways in cancer is the DNA Damage Response (DDR), a well-coordinated network of signalling cascades that cells have developed during evolution in order to maintain genomic integrity. These pathways are activated when genomic material is damaged as a consequence of exposition to deleterious endogenous and environmental genotoxic agents, as well as the intrinsic chemical instability of the DNA molecule itself (Hanawalt, 2015), with the aim of transmit the damage signals to effector proteins, and induce cellular responses including cell cycle arrest, activation of DNA repair pathways, and cell death (Ciccia & Elledge, 2010). When the DNA-damaging agents cause chromosomal instability in essential 'driver' genes, the cell behavior can be altered, conferring a selective advantage and driving the development of cancer. Notably, it also can determine the response to therapy of the tumor (Lord & Ashworth, 2012).

3.1.1. Mechanism of DNA Damage Response

Endogenous DNA damage is caused by reactive oxygen species (ROS) generated spontaneously during DNA metabolism, especially those arising from the process of oxidative deamination and from replication errors (they can be due to DNA bases interconversion, dNTP misincorporation, loss of DNA bases or modification of DNA bases by alkylation). Environmental DNA damage can be produced by physical or chemical sources. Examples of physical genotoxic agents are ionizing radiation (IR), ultraviolet (UV) light and certain plant toxins. Chemical agents are those used in cancer chemotherapy (human-made mutagenic chemicals, especially aromatic compounds),

without forgetting the smoke derived from cigarettes (Ciccia & Elledge, 2010; Tian *et al.*, 2015).

These DNA-damaging agents can induce different types of DNA lesions including modification of bases, intrastrand or interstrand crosslinks (ICL), DNA–protein crosslinks, single-strand breaks (SSB) and double-strand breaks (DSB). Each type of DNA damage is recognized and processed by proteins involved in the DDR pathways, that can either work independently or coordinately to repair the lesions (Hosoya & Miyagawa, 2014). In any case, most of them encompass a similar set of tightly coordinated processes: DNA damage detection, DNA repair factors recruitment to the onset of the lesion and the final restore of the damage (Lord & Ashworth, 2012).

Under normal physiological conditions, there are seven major mechanisms entrusted of restoring the diverse types of DNA damage generated: base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end joining (NHEJ), Mismatch repair (MMR), activation of the Fanconi anemia (FA) pathway and translesion DNA synthesis (TLS). BER proteins repair most of the DNA subtle changes, such as oxidative lesions, alkylation products and SSBs. Poly (ADP-ribose) polymerase (PARP-1), XRCC1, DNA ligase IIIa, and apurinic / apyrimidinic endonuclease (APE1) are examples of BER proteins (Dianov & Hubscher, 2013). However, some of the bulkier single-strand lesions that distort the DNA helical structure, such as pyrimidine dimers caused by UV irradiation are processed by the NER pathway (Cleaver *et al.*, 2009; Kamileri *et al.*, 2012), which requires the excision repair cross-complementing protein 1 (ERCC1). DSBs are mainly restored either by HR, through the MRN complex and the proteins CtIP, replication protein A (RPA), BRCA1, BRCA2, RAD51 and PALB2 (Hartlerode & Scully, 2009; Moynahan & Jasin, 2010), or NHEJ processes. Factors involved in NHEJ include the Ku70 / Ku80 complex, DNA-PK catalytic subunit (DNA-PKcs), the Artemis nuclease, XLF, XRCC4, and DNA ligase IV (Lieber, 2010; Zimmermann & de Lange, 2014). Replication errors results in base mismatches, which are repaired through the so-called MMR pathway (Hsieh & Yamane, 2008), essentially managed by proteins encoded by *MSH2* and *MLH1* genes. The ubiquitin-mediated activation of the FA pathway plays a key role in restoring the

intrastrand or interstrand crosslinks. Finally, translesion synthesis and template switching allow DNA to continue to replicate in the presence of DNA lesions that would otherwise halt the process. Therefore, both mechanisms are usually considered to be part of the DDR. The mechanisms that control the integrity of telomeric DNA in human chromosomes constitute a barrier against mutations and genome instability, although are not considered to belong to the DDR by some authors (Artandi & DePinho, 2010).

Cell-cycle-checkpoint and chromosome-segregation machineries interact with core components of the DDR, thus facilitating the DNA repair to occur before mitosis and ensuring the correct genetic material segregation to daughter cells (Hanahan & Weinberg, 2011) (Figure 1).

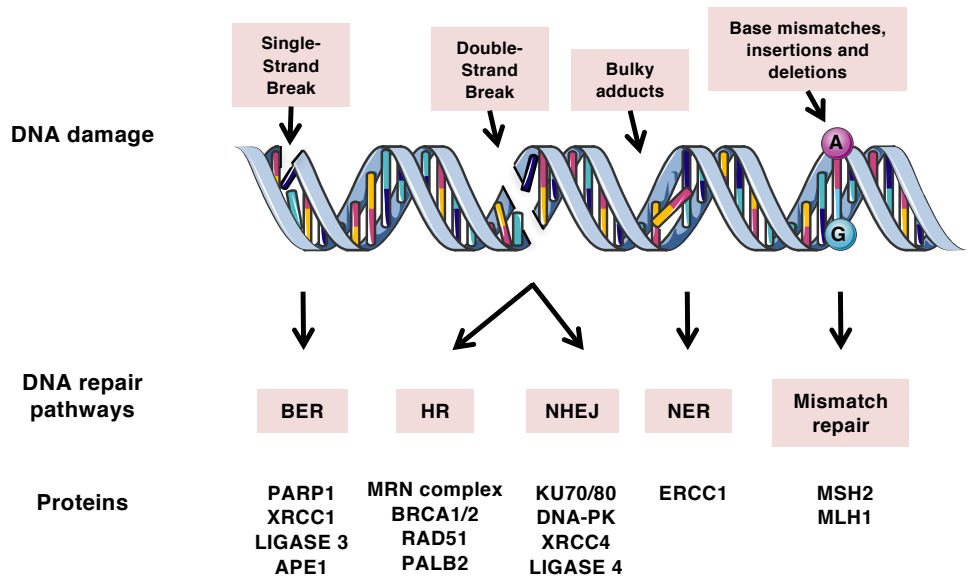

Figure 1. Overview of the diverse spectrum of DNA damage and the DNA damage response. The major repair pathways and key proteins used to process each type of damage are shown. BER: base excision repair; NER: nucleotide excision repair; HR: homologous repair; NHEJ: non-homologous end-joining (*Adapted from Lord et al, Nature, 2012).*

DSBs are among the most deleterious DNA lesions, which if unrepaired or repaired incorrectly can cause cell death or genome instability that may lead to cancer. Within the homologous recombination response, the MRE11–RAD50–NBS1 (MRN) complex is recruited to the damaged DNA sites, and phosphorylates a variant H2A

histone (H2AX) (Fernandez-Capetillo *et al.*, 2004), thus intensifying the signal and recruiting new molecules to the DSB lesion, where lastly the ATM (ataxia-telangiectasia mutated) kinase is activated. MRN complex itself is also capable of phosphorylating ATM (Bakkenist & Kastan, 2003; Falck *et al.*, 2005; Lee & Paull, 2005). Once activated, ATM orchestrates a large number of downstream effectors (Matsuoka *et al.*, 2007). Phosphorylation of CHK2 (checkpoint kinase 2) on Thr68 inactivates members of the CDC25 family of dual-specificity phosphatases, responsible for ruling cell cycle divisions (Donzelli & Draetta, 2003), and consequently leads to cell cycle arrest. ATM-dependent phosphorylation of the tumor-suppressor BRCA1, as well as its inhibitor CtIP, leads to DSB repair and cell cycle arrest in the S phase (Cortez *et al.*, 1999) (Gatei *et al.*, 2001). In this regard, CHK2, previously activated by ATM, likewise phosphorylates and activates BRCA1. ATM also phosphorylates and activates p53 (Ser15), what triggers cell cycle arrest in the G1 phase or cell death.

As a final result, p53 Ser46 phosphorylation mediated apoptosis will be achieved by a number of kinases such as ATM, DNA-PK, PKCδ, DYRK2 and HIPK2 (D'Orazi *et al.*, 2002; Komiyama *et al.*, 2004; Saito *et al.*, 2002; Taira *et al.*, 2007; Yoshida *et al.*, 2006). DYRK2 (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2) is regulated by Mdm2 under normal conditions (Taira *et al.*, 2010). Upon genotoxic stress, both proteins dissociate due to a direct phosphorylation on DYRK2 (Thr33 and Ser369) by ATM. Once this occurs, DYRK2 phosphorylates p53 on serine 46 and the apoptotic response finally takes place (Figure 2).

3.1.2. Aberrations in DNA Damage Responses and Human Disease

DNA damage response in human physiology plays a central role as can be proved from the broad spectrum of defects displayed by individuals carrying mutations in DDR genes. DDR genetic syndromes primarily produce neurological defects, infertility, immunological defects, premature aging, stem cell exhaustion and cancer development (Ciccia & Elledge, 2010).

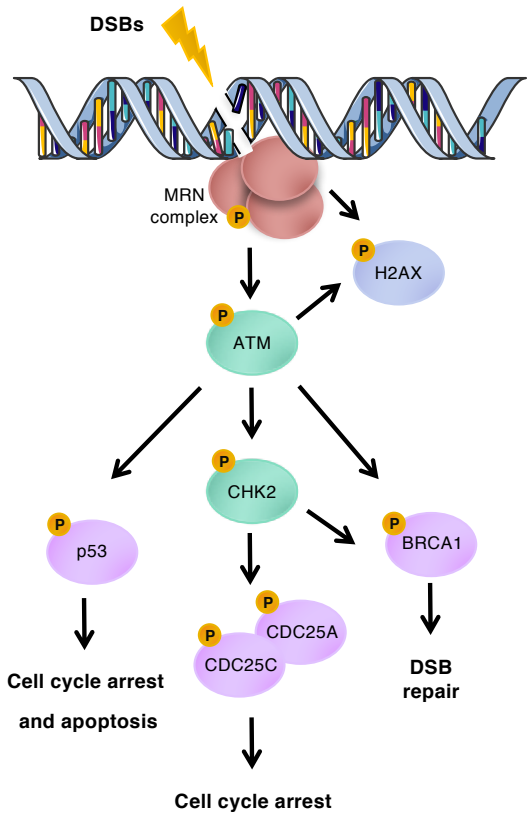

Figure 2. DNA damage response (DDR) pathway in response to DNA double-strand breaks (DSBs), trough MRN complex sensor (Adapted from *Stolz et al, Clin Cancer Res, 2011* and *Sulli et al, Nat Rev Cancer, 2012).*

Maintenance of genomic integrity by the DDR is critical to prevent tumorigenesis, as indicated by the cancer-prone phenotype of several DDR syndromes, such as XP syndrome or Bloom syndrome (BS), where DDR alterations increase the risk of skin cancer and melanoma or lymphomas, leukemias and carcinomas, respectively (Ciccia & Elledge, 2010; Negrini *et al.*, 2010).

Dysfunctionality of the DDR is noticeable at an early stage in the development of neoplasia. Markers of DSBs, such as nuclear γH2AX foci (a histone phosphorylation event that occurs on chromatin surrounding a DSB), are markedly elevated in some precancerous lesions (Bartkova *et al.*, 2006; Halazonetis *et al.*, 2008). For

precancerous lesions to progress to mature tumors, it is thought that critical DSB and cell-cycle checkpoint proteins, such as ATM, ATR (ataxia telangiectasia and Rad3-related protein), and the master 'gatekeeper' protein p53 become inactivated, due to mutations, deletion or loss of heterozygosity of the genes (Brosh & Rotter, 2009; Hosoya & Miyagawa, 2014). This leads to an ineffective DNA damage repair before mitosis and cells proceed through the cell cycle with DNA lesions intact, increasing the chance of mutagenesis (Lord & Ashworth, 2012).

Nevertheless, controversy exists regarding the expression of DDR proteins in tumors, as both activation and inactivation are observed in a large spectrum of sporadic cancers. DDR proteins activation may serve as a barrier to the malignant progression and metastasis. For instance, augmented autophosphorylation of ATM and ATM-dependent phosphorylation of CHK2 are reported in early-stage tumors (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005), and hyper activation of ATM has been observed in late-stage breast tumor tissues (Sun *et al.*, 2012). Increased expression of a large list of other DDR proteins (NBS1, RAD50/51, CHK1/2, CDC25s, DNA-PK, BRCA1, ERCC1, APE1, and PARP1) has been also reported in various cancers, being in some cases associated with resistance to chemotherapy (Abbotts & Madhusudan, 2010; Hannay *et al.*, 2007; Mego *et al.*, 2013; Olaussen *et al.*, 2006; Takenaka *et al.*, 2007; Taron *et al.*, 2004). On the contrary, inactivation of some of the previous proteins (ATM, MRN complex, CHK2, RAD51, BRCA1/2, and ERCC1) is also frequently observed in other cohort of tumors. These data indicate the common aberration of the DDR is evident in sporadic cancers, presenting an important role in the development of the disease (Hosoya & Miyagawa, 2014).

3.1.3. Targeting DNA Damage Response Pathways for Cancer Therapy

DDR defects have already been exploited in cancer treatment, being the targets of drugs commonly used in chemotherapy and radiotherapy. One example is the use of platinum salts (carboplatin, cisplatin, oxaliplatin), that induce covalent crosslinks between DNA bases. An alternative approach to chemotherapy is the design of drugs that target specific DDR components, such as agents that cause SSB or DSB by

trapping topoisomerase I or II enzymes on DNA (camptothecin or irinotecan and etoposide, respectively) (Ciccia & Elledge, 2010). These topoisomerase inhibitors could be regarded as the first generation of DDR 'targeted' agents (Pommier *et al.*, 2010). Within the next generation of DDR inhibitors, PARP1 inhibitors as targeted therapy are included. They have been successfully used to treat tumors that carry mutations in HR genes, such as *BRCA1* and *BRCA2* (Jackson & Bartek, 2009).

Furthermore, given the elevated levels of DNA damage in cancer cells compared to healthy ones, one approximation as selective anticancer therapy is further increase the amount of DNA damage (by inhibition of DDR components) in combination with other chemotherapeutic drugs, thus augmenting cancer cell death. For instance, it would be interesting, in order to kill the malignant cells and not the normal ones, to block the alternative repair pathway that is activated in cancer cells to partially compensate their already disrupted DNA repair pathway. This is the principle of action of PARP inhibitors commented before, which if in addition are combined with radiotherapy, effectively prohibit tumor malignant transformation through the synergistic effect. In order to further disrupt the DNA repair pathway responsible for the tumor survival and drug resistance, one potential treatment considered is to target the cell cycle checkpoint by inhibiting ATM (the predominant kinase involved in the activation of the response to DSB) or CHK1 and CHK2. Indeed, it has been proved the preferential toxicity of ATM, DNA-PK and CHK1 inhibitors towards cancer cells after treatment with genotoxic agents (Bolderson *et al.*, 2009). Interestingly, ATM and DNA-PK depletion have been shown to sensitize *TP53* and *ATM* mutant tumor cells to genotoxic agents, respectively (Jiang *et al.*, 2009). Emerging evidence suggests that CHK1 and CHK2 inhibitors may have high antitumor properties due to their key roles in the specific regulation of checkpoint control of the cells. This suggest a further advantage of inhibiting the checkpoint kinases over their upstream activators ATM and ATR involved additionally in the activation of DNA repair (Tse *et al.*, 2007; Welch *et al.*, 2007). AZD7762, XL844 and PF-47736 combined with other DNA damaging agent exert enhanced cytotoxicity toward malignant tumor cells (Janetka *et al.*, 2007). Regarding p53, the blockade of its function (that leads to G2 checkpoint abrogation) can sensitize

cancer cells to cytotoxic agents as it has been demonstrated in squamous cell carcinoma of the head and neck in response to radiotherapy (Tian *et al.*, 2015).

3.2. CHK2

3.2.1. CHK2 family context

As mentioned above, the serine threonine kinase CHK2 is a highly conserved protein critical in the DDR signaling pathway, which is activated to protect the cells against nuclear DNA alterations (Stracker *et al.*, 2009; Zannini *et al.*, 2014). CHK2 is a CAMK protein kinase of the Rad53 family, whose structure is similar in all eukaryotes. Human, mouse, rat, zebra fish and *C. elegans* homologues of the protein kinase are called CHK2, whereas the homologues in other species present alternative names: Xcds1 (*X. laevis*), cds1 (*S. pombe*), Rad53 (*S. cerevisiae*) and Dmnk (*D. melanogaster*). Rad53 was the founding member of the “CHK2 family” of check-point kinases and was first identified in 1994 (Bartek *et al.*, 2001).

3.2.2. Structure and function of CHK2

Human CHK2 protein, encoded by Chk2 gene located on chromosome 22q12.1, presents 543 residues and contains three different functional domains: an N-terminal region rich in serine-glutamine and threonine-glutamine pairs, called SQ/TQ cluster domain (SCD, residues19–69), which satisfy the primary substrate motif for ATM/ATR kinases, especially residues 67–70; a forkhead-associated domain (FHA, residues 113–175) responsible for interactions with other proteins in *trans* and for affecting functional domains within the protein itself in *cis*; and a canonical kinase domain (residues 220–486) that occupies almost the entire C-terminal half of CHK2, containing Asp as a catalytic residue at the active site and an activation loop within its key functional elements (Ahn *et al.*, 2004; Buscemi *et al.*, 2004; Li *et al.*, 2002a; Matsuoka *et al.*, 2007) (Figure 3).

Upon activation, CHK2 phosphorylates and regulates several proteins involved in different steps of the DDR, such as DNA repair, cell cycle regulation, apoptosis and p53 signaling (Antoni *et al.*, 2007). To date, 24 substrate proteins of this kinase have been described in human cells, amongst which BRCA1, CDC25A and CDC25C, HDMX, PML (Promyelocytic Leukemia) and E2F1 are included (Chaturvedi *et al.*, 1999; Pereg *et al.*, 2006; Stevens *et al.*, 2003; Yang *et al.*, 2002; Zannini *et al.*, 2014; Zhang *et al.*, 2004). Interestingly, both BRCA1 and PML substrates interacts with CHK2 in the absence of DNA damage but, following gamma irradiation, BRCA1 is phosphorylated at Ser988 and PML at Ser117 in a CHK2-dependent manner, resulting in subsequent inhibition of binding with CHK2 and leading to the induction of DNA repair (Ahn *et al.*, 2004; Zhang *et al.*, 2004). Regarding cell cycle regulation, CHK2 phosphorylates CDC25A (Ser123) and CDC25C (Ser216) phosphatases after DNA damage, thus mediating CDC25A $SCF^{\beta TCRP}$-dependent turnover and persistent cytoplasmic CDC25C localization that prevents the G2/M transition. Accordingly, CHK2 phosphorylation of these phosphatases induces cell cycle arrest in response to DNA damage (Ahn *et al.*, 2004; Takai *et al.*, 2002). CHK2 phosphorylates E2F-1 at Ser364 in response to the DNA-damaging agent etoposide (ETP), regulating its stabilization and transcriptional activity, what represents a potentially relevant pathway through which CHK2 regulates DNA damage-induced apoptosis (Stevens *et al.*, 2003). Importantly, CHK2 presents the ability to regulate p53 activity, either directly through Ser20 phosphorylation (Chehab *et al.*, 2000), or indirectly phosphorylating other proteins, such as Che-1, STRAP, HDMX (Choi *et al.*, 2013; Zhang *et al.*, 2004). Ser20 phosphorylation by CHK2 stabilizes p53 protein, enhancing its potential to positively regulate the expression of factors that are involved in DNA repair, cell death and cell-cycle control. Likewise, p53 phosphorylation by CHK2 at residues Ser366 and Thr387 has been described, being involved in cell cycle arrest at G1/S and G2/M and in p53 acetylation regulation, respectively (Bruno *et al.*, 2006). Several substrates of CHK2 (p53, PML, E2F1, BRCA1, and CHK2 itself) are also proteins phosphorylated by ATM, reflecting the possible redundant role of both proteins and suggesting that CHK2 can act as a relay for ATM and/or as a salvage pathway when ATM is inactivated (Matsuoka *et al.*, 2007; Wang *et al.*, 2004; White *et al.*, 2006).

3.2.3. Localization and expression of CHK2

CHK2 is predominantly -if not exclusively- located in the nucleus of mammalian cells, what was expected for a protein involved in the regulation of the DNA-damage checkpoint. CHK2 often localizes distinct subnuclear bodies or 'foci', and, indeed, ionizing radiation triggers its accumulation at sites of DNA strand breaks (upon DNA damage, activated Thr68-CHK2 colocalizes with 53BP1, H2AX, and NBS1 in nuclear foci). However there is an intriguing localization of CHK2 in human neuronal cells, where it has a predominantly cytoplasmic localization, in contrast to other cell and tissue types. ATM–CHK2 pathway in neurons might have a specialized cytoplasmic role possibly related to protection from against oxidative stress. In fact, patients with ataxia-telangiectasia, who lack functional ATM, suffer from severe neurodegeneration, which probably reflects the elevated oxidative stress and progressive neuronal cell death. Although CHK2 is mainly nuclear in somatic cells, several reports document the presence of a subpopulation of CHK2 at centrosomes. Strikingly, in embryonic stem cells, CHK2 has been shown to localize exclusively at the centrosomes as well as in *Drosophila melanogaster* embryos (DmCHK2 homologue). On the other hand, Chouinard and colleagues demonstrated that CHK2 localizes exclusively in the nucleus of cells in interphase and that a small portion was localized to centrosomes in mitotic cells (for what PLK1 activity was required), from late prophase until cytokinesis supporting a role for CHK2 during mitosis (Chouinard *et al.*, 2013).

Studies of mammalian CHK2 messenger RNA expression indicated a broad but variable CHK2 distribution, with the highest levels in the testis, bone marrow and immune system and gastrointestinal tract. According to its function, CHK2 is generally expressed in cells of proliferating, as well as terminally differentiated, non-proliferating tissue compartments (Bartek *et al.*, 2001). Regarding protein expression, the kinase is markedly present in almost all human tissues with the exception of adipose and soft tissue, and a low expression exists in brain or muscle tissues. Generally, tumor tissues show a higher expression of CHK2 compared with its respective non-tumor one. Most cancer tissues presents moderate to strong nuclear staining, but several cases of renal cancers are negative or weakly stained (Protein Atlas).

3.2.4. CHK2 regulation

CHK2 exists as an inactive monomer in unperturbed cells. Following DNA damage, mainly DSB, CHK2 undergoes dimerization and consequently, becomes active. In order to achieve the kinase dimerization, several phosphorylation steps are needed. This first activating phosphorylation step of CHK2 is performed by ATM at Thr68, and depends on the integrity of its FHA domain, since one P-Thr68-CHK2 molecule binds to the FHA domain of second molecule. Phosphorylation at Thr68 is the initiating event for dimerization and activation since its maintenance is not required for sustained kinase activity. The subsequent activation step is attributable to autophosphorylation of CHK2 on residues Thr383 and Thr387 within the auto-inhibitory loop, resulting in kinase activation. An extra autophosphorylation event at Ser516 (C-terminal) is required for full kinase activation (Ahn *et al.*, 2004; Bartek *et al.*, 2001; Guo *et al.*, 2010a; Lovly *et al.*, 2008) (Figure 3).

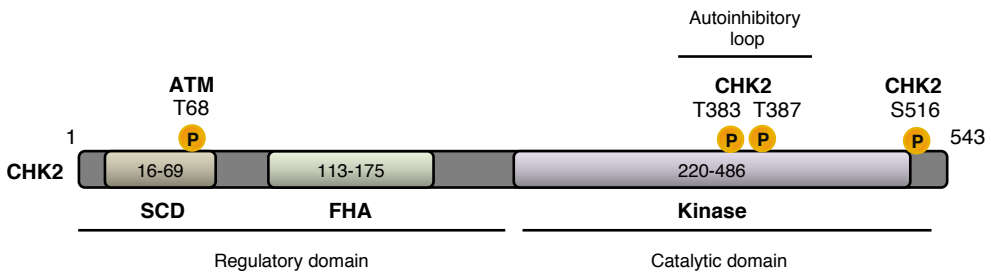

Figure 3. **Scheme of CHK2 protein structure**. SCD, FHA and Kinase domains are included. Phosphorylated threonine (T) and serine (S) residues needed for CHK2 activation are shown.

Although CHK2 activation control mechanisms have been widely described, very little is known about its inactivation and degradation processes. Serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) and protein phosphatase 1D (WIP1) have been characterized as responsible to inactivate CHK2. Under normal conditions, B'α subunit of PP2A binds to the SQ/TQ repeat region of CHK2 and negatively regulates the kinase through its de-phosphorylation at multiple sites (Ser19, 33, and 35 and Thr68); therefore modulating its kinase activity over substrates such as CDC25C. However, the interaction of both proteins is disrupted after DNA damage. PP1

phosphatase cooperates with PP2A to maintain CHK2-T68 unphosphorylated in undamaged cells (Carlessi *et al.*, 2010; Freeman *et al.*, 2010). On the other hand, WIP1 association with CHK2 inhibits its Thr68 phosphorylation and consistent activation under DNA damage, resulting in the repression of CHK2 kinase activity toward CDC25C (Fujimoto *et al.*, 2006).

Likewise, during the last decade several evidences have suggested that ubiquitination is an important control process in CHK2 turnover. The ubiquitin protease USP28 regulates the CHK2-p53-PUMA pathway; major regulator of DNA-damage-induced apoptosis in response to DSB. USP28 is required to stabilize both CHK2 and 53BP1 in response to DNA damage (Zhang *et al.*, 2006). CHK2 has been reported as a new substrate of the E3 ubiquitin ligase RING finger protein 8 (RNF8) in humans, which ubiquitinates CHK2 diminishing this way its abundance and activation in response to ionizing radiation (Feng & Chen, 2012). Finally, it has been demonstrated that p53-induced RING-H2 protein (PIRH2) is the E3 ubiquitin ligase of CHK2 in mice. PIRH2 interacts with CHK2 and mediates its polyubiquitylation and proteasomal degradation, thus regulating its turnover and its function. Interestingly, USP28 forms a complex with PIRH2 and CHK2 and antagonizes PIRH2-mediated polyubiquitylation and proteasomal degradation of CHK2 (Bohgaki *et al.*, 2013).

Even though the recent identification of these enzymes as regulators of CHK2 through ubiquitination in response to DNA damage, the mechanisms required to maintaining the steady-levels of such a relevant protein remain poorly understood.

3.2.5. SIAH2

The E3 ubiquitin ligase SIAH2 (seven in absentia homolog 2) belongs to the RING finger E3 ubiquitin ligases (Hu *et al.*, 1997; Schnell & Hicke, 2003). In humans, two subunits (SIAH1 and SIAH2) have been described, which are codified by different genes and present similar and redundant functions (Della *et al.*, 1993; Holloway *et al.*, 1997). Human SIAH2 is a 324 amino acid dimeric protein that mediates ubiquitination and subsequent proteasomal degradation of target proteins. It presents a RING (Real

Interesting New Gen) catalytic domain in its N-terminal that recruits E2 ubiquitin conjugating enzymes, two zinc-finger motifs and a substrate-binding domain (SBD) in its C-terminal (House *et al.*, 2009; Polekhina *et al.*, 2002) (Figure 4A).

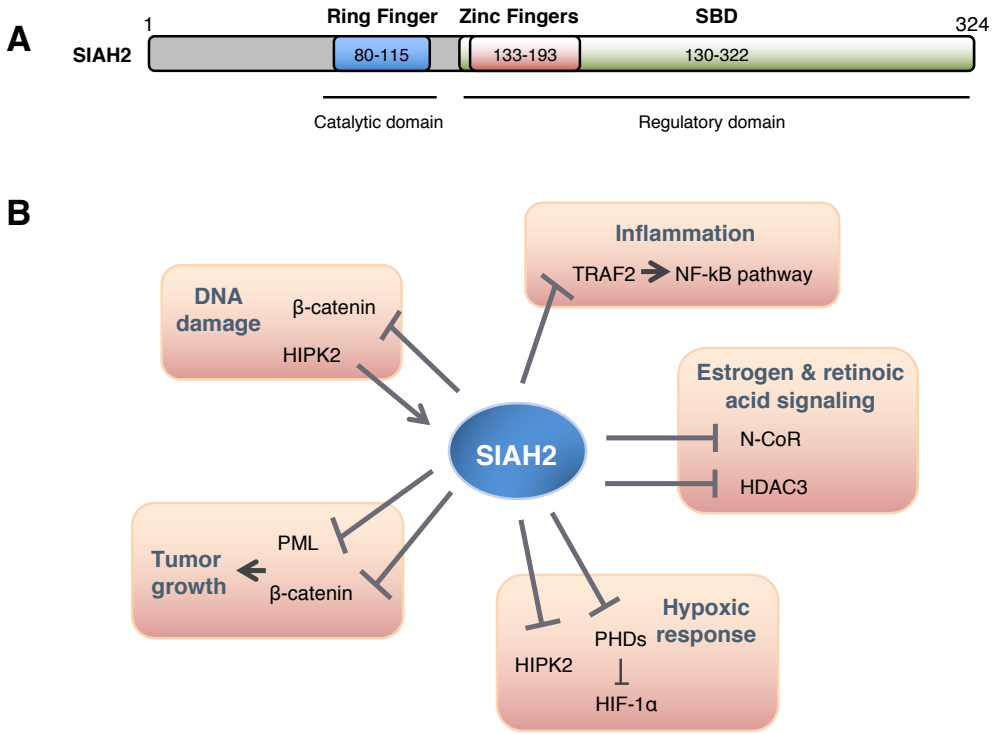

Figure 4. SIAH2 protein structure and function. A. SIAH2 full sequence showing the three basic domains: RING, Zinc Fingers and SBD. **B.** Schematic overview of SIAH2 function trough some substrates.

SIAH2 is responsible for ubiquitination and proteasomal degradation of specific substrates, either through direct interaction or through binding to adapter proteins (Calzado *et al.*, 2009; Li *et al.*, 2002b; Matsuzawa & Reed, 2001; Sarkar *et al.*, 2012). SIAH2 controls the expression of substrates involved in crucial processes such as apoptosis, tumor suppression, cell cycle, transcription and cell signaling. Within the most relevant SIAH2 substrates described to date PML, N-CoR, HDAC3, TRAF2, β-catenin, HIPK2 and DYRK2 are included. SIAH2 binds and targets for proteasome-mediated degradation the putative tumor suppressor PML (Promyelocytic leukemia protein), leading to the loss of its transcriptional co-activating properties and a reduction

in the number of endogenous PML nuclear bodies (Fanelli *et al.*, 2004). SIAH2 also interacts with N-CoR, TRAF2, β-catenin and HDAC3, mediating their proteasomal degradation (Habelhah *et al.*, 2002; Topol *et al.*, 2003; Zhang *et al.*, 1998; Zhao *et al.*, 2010). Two members of the dual-specificity tyrosine-regulated kinase (DYRK) family of kinases, HIPK2 and DYRK2 have been previously described by our group as SIAH2 substrates, existing in both cases a mutual regulation between the ligase and the kinase, wherein the kinases also phosphorylate SIAH2 regulating its function (Calzado *et al.*, 2009; Perez *et al.*, 2012). However the key role of SIAH2 on the regulation of hypoxia response stands out, mainly due to its ability to alter the expression of hypoxia-inducible transcription factor 1 alpha (HIF-1α), which occurs through the degradation of the Hypoxia-inducible factor prolyl hydroxylases (PHDs), (Appelhoff *et al.*, 2004; Le Moan *et al.*, 2011; Nakayama *et al.*, 2004) (Figure 4B).

SIAH2 expression can be controlled at a transcriptional level by estrogens, different transcription factors (WNT5a and E2F1) and small noncoding microRNA (MiR-146b) (Frasor *et al.*, 2005; Knauer *et al.*, 2015; Liao *et al.*, 2013; Topol *et al.*, 2003; Xie *et al.*, 2009). Different post-translational modifications able to modify SIAH2 activity have been described, such as their ability to autoubiquitinate and the phosphorylation mediated by upstream kinases (Hu & Fearon, 1999). To date, only 3 kinases able to phosphorylate SIAH2, modifying its activity, have been described: p38 MAPK, HIPK2 and DYRK2. p38 MAPK phosphorylates murine SIAH2 in Thr24 and Ser29 increasing SIAH2-mediated degradation of PHD3 (Khurana *et al.*, 2006). In this sense, phospho-mutant SIAH2 shows weaker association with PHD3, while phospho-mimic SIAH2 associates as the wild type does and is localized within the perinuclear region, suggesting that phosphorylation of SIAH2 affects its subcellular localization and, therefore, the degree of its association with its substrates. Likewise, the serine/threonine kinase HIPK2 is described to phosphorylate human SIAH2 in Thr26, Ser28 and Ser68 thus negatively affecting its stability and weaking its interaction with HIPK2 (Calzado *et al.*, 2009). The highly related HIPK2 protein, DYRK2, has also been identified as SIAH2 interaction partner that phosphorylates it in a minimum of five residues (Ser16, Thr26, Ser28, Ser68, and Thr119), leading to an increase in the SIAH2 ability to trigger the HIF-1a-mediated transcriptional response and angiogenesis

(Perez *et al.*, 2012). On the other hand, the deubiquitinating enzyme USP13 increases SIAH2 stability by attenuating its activity to degrade both itself and its substrates prolyl hydroxylase 3 and Spry2 (Sprouty2). It is worth noting that in melanoma cancer cells USP13 levels are decreased under hypoxia thereby relieving SIAH2 inhibition and increasing its activity (Scortegagna *et al.*, 2011).

3.3. DYRK2

3.3.1. DYRK family

The Dual-specificity tyrosine-regulated (DYRK) family of kinases belongs to the CMGC group, and is divided in turn into three subfamilies according to the homology within their kinase domain: DYRK kinases, homeodomain-interacting protein kinases (HIPKs), and pre-mRNA processing protein 4 kinases (PRP4s). DYRK subfamily members have been found in all eukaryotes and share common structural, biochemical, and functional properties with their ancestors in yeast (the founding member Yak1p was discovered in budding yeast more than 20 years ago). In mammals, DYRK subfamily comprises DYRK1A and DYRK1B (class I), DYRK2, DYRK3, and DYRK4 (class II). Homologues of DYRK1A and DYRK1B have been reported in *Caenorhabditis elegans* (MBK-1 and MBK-2), whereas homologues of class II DYRKs exist in *Drosophila melanogaster* (minibrain, dDyrk2, and dDyrk3). The five members of the subfamily share the kinase domain as well as a sequence just upstream of the kinase domain named the DYRK homology (DH)-box, what reflects the conservation among the different members at the protein level (Aranda *et al.*, 2011).

DYRK1A, founding member of the family, is the best well known among the rest isotypes and maps to the DSCR (Down Syndrome Critical Region) of chromosome 21 (Guimera *et al.*, 1996; Shindoh *et al.*, 1996; Song *et al.*, 1996). Besides being involved in neurological disorders such as Down syndrome, it is also implicated in calcium signaling by phosphorylating NFAT (Gwack *et al.*, 2006) and cell polarity maintenance in *Schizosaccharomyces pombe* (Tatebe *et al.*, 2005). Furthermore it is embroiled in

various biochemical pathways as it is able to phosphorylate many other substrates such as: STAT3 (Matsuo *et al.*, 2001), eIF2B and Tau (Woods *et al.*, 2001). DYRK1B mediates cell survival and differentiation and is highly expressed in muscle tissues (Lee *et al.*, 2000; Mercer *et al.*, 2005; Mercer & Friedman, 2006). It has been reported to phosphorylate among other substrates p21Cip1 (Zhou *et al.*, 2001) and class II HDACs (Deng *et al.*, 2005). DYRK3 is highly produced in hematopoietic cells and testis. It reduces apoptosis in response to cytokine starvation in hematopoietic cells (Geiger *et al.*, 2001) and has been shown to phosphorylate SIRT1 (Guo *et al.*, 2010b), myelin basic protein and histones H2b and H3 (Lord *et al.*, 2000). DYRK4 is the least known isoform of all the DYRK family members. It is exclusively expressed in the testis and helps the process of spermiogenesis (Sacher *et al.*, 2007).

3.3.2. Structure and function of DYRK2

Human DYRK2, encoded by Dyrk2 gene located on chromosome 12q15, is a 528 amino acid (aa) protein that contains the following functional domains: first, as the rest of the members of its subfamily, a kinase domain (residues 149-462) and a DH box necessary for the formation of tertiary structure in the N-terminus; additionally, it presents two functional domains, the N-terminal autophosphorylation accessory regions 1 and 2 (NAPA1 and NAPA2), shared by class II DYRK proteins, and a nuclear localization signal (NLS) (Becker *et al.*, 1998; Soundararajan *et al.*, 2013) (Figure 5A). Two isoforms of human DYRK2 resulting from 5'-UTR alternative splicing have been reported, which differ in their N-termini domain (short isoform: 528 aa, long isoform: 601 aa).

DYRKs are defined as dual-specificity protein kinase because they have tyrosine phosphorylation activity restricted to autophosphorylation so as to become active and serine/threonine phosphorylation activity on exogenous substrates. Indeed, no phosphorylated tyrosines have been found in any DYRKs substrate to date. DYRKs require phosphorylation of the activation loop (with a conserved YXY sequence; Y307-T-Y309 in the case of DYRK2) to achieve full activity. This autocatalytic process occurs during the translation of the protein, when a transient intermediate form, helped by the

NAPA domain that provides a chaperone-like function, is auto phosphorylated at the second tyrosine within the activation loop. The specificity of this intermediate for tyrosines is lost on completion of translation, and kinase activity becomes restricted to threonine and serine (Han *et al.*, 2012; Kinstrie *et al.*, 2010; Lochhead *et al.*, 2005).

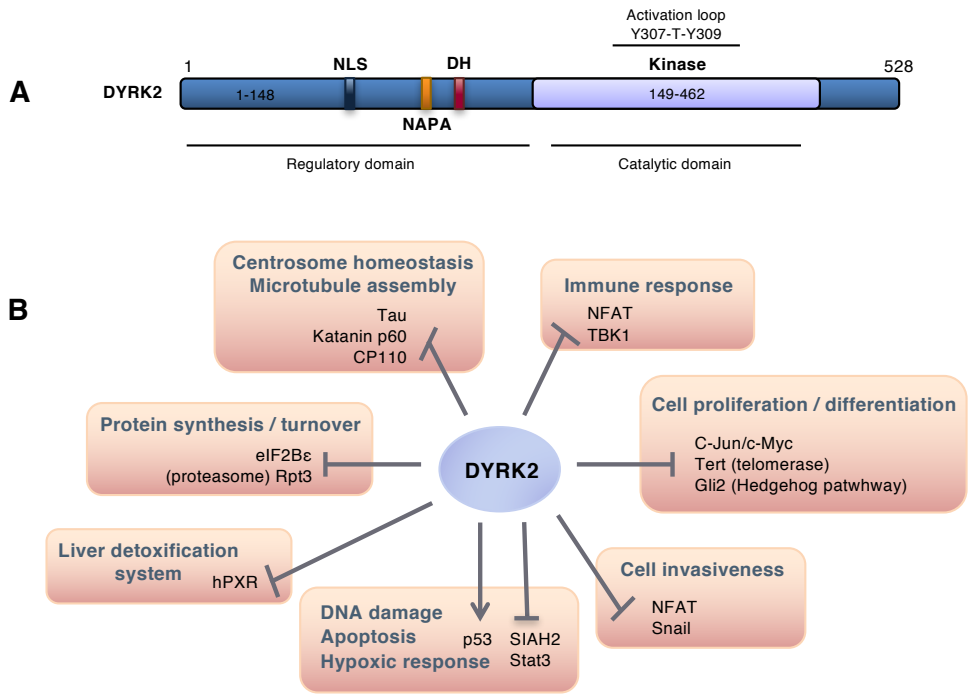

Figure 5. DYRK2 protein structure and function. A. Representation of DYRK2 structure, showing the functional domains. **B.** Schematic overview of DYRK2 function trough some substrates.

DYRK2 is involved in regulating key developmental and cellular processes such as neurogenesis, cell proliferation, cytokinesis, and cellular differentiation. Substrates for DYRK2 by serine/threonine phosphorylation share consensus phosphorylation sites: R/Kxx(x)**S/T**P/V, wherein S or T is usually followed by a proline (Gwack *et al.*, 2006; Maddika & Chen, 2009; Yoshida, 2008). Despite the crucial role of this kinase, especially in tumor development and progression, there are only 18 DYRK2 substrates described to date: Stat3 (Matsuo *et al.*, 2001), eIF2Bε, Tau (Woods *et al.*, 2001), Glycogen synthase (Skurat & Dietrich, 2004), NFAT (Gwack *et al.*, 2006), SNR1 (Kinstrie *et al.*, 2006), p53 (Taira *et al.*, 2007), Gli2 (Varjosalo *et al.*, 2008), Katanin p60

(Maddika & Chen, 2009), c-Jun and c-Myc (Taira *et al.*, 2012), SIAH2 (Perez *et al.*, 2012), Tert (Jung *et al.*, 2013), hPXR (Ong *et al.*, 2014), Snail (Yamaguchi *et al.*, 2015), TBK1 (An *et al.*, 2015), Rpt3 (Guo *et al.*, 2016) and CP110 (Hossain *et al.*, 2017) (Figure 5B).

Several of the prior DYRK2 substrates are involved in cancer processes. Ten years ago, a finding of great importance described a major role of DYRK2 as concern the regulation of cell physiology upon DNA damage: DYRK2-dependent p53 phosphorylation at Ser46, which leads to the induction of cellular apoptosis (Taira *et al.*, 2007). The important cell cycle regulators c-Jun and c-Myc are also phosphorylated by DYRK2, leading to is proteasomal degradation in the G1/S transition. Therefore, the maintenance of the steady-levels of these transcription factors by DYRK2 prevents an aberrant cell proliferation (Taira *et al.*, 2012). Our group previously demonstrated that SIAH2 is a substrate for DYRK2 phosphorylation, taking place a mutual regulation between both enzymes under hypoxia and genotoxic stress (Perez *et al.*, 2012). Moreover, it has been reported a relevant role for DYRK2 regarding proliferation and invasion of cancer cells, by the regulation of the Tert subunit of the Telomerase (Jung *et al.*, 2013) and Snail (Mimoto *et al.*, 2013; Yamaguchi *et al.*, 2015), respectively. Recently, it has been described that the proteasome is dynamically phosphorylated during the cell cycle at Thr25 of the 19S subunit Rpt3 by DYRK2, leading to enhanced substrate translocation and degradation what finally results in breast tumor formation in mice (Guo *et al.*, 2016; Huibregtse & Matouschek, 2016). These findings demonstrate the biological significance of DYRK2 function in regulating cell proliferation and tumorigenesis (Figure 6).

3.3.3. Localization and expression of DYRK2

DYRK2 has a cytoplasmic subcellular localization under normal conditions (Becker *et al.*, 1998; Leder *et al.*, 1999), although it accumulates in the nucleus in response to genotoxic stress (Taira *et al.*, 2007). This nuclear accumulation depends on the NLS domain of the kinase and on a release from Mdm2 degradation (Taira *et al.*, 2010).

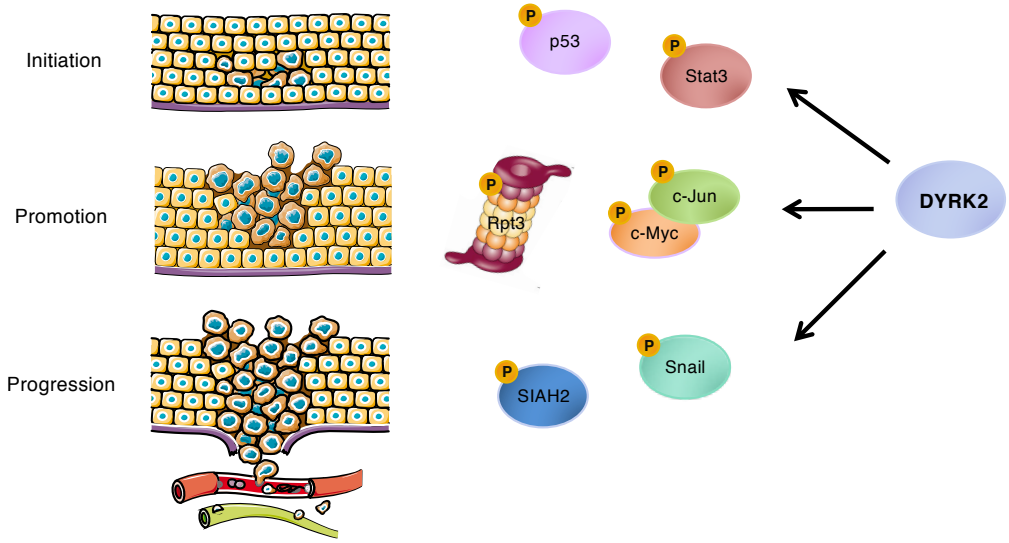

Figure 6. DYRK2 role in cancer. Schematic overview of DYRK2 involvement in the different phases of the carcinogenic process.

Studies of DYRK2 messenger RNA expression indicated a low expression in human tissues, with the exception of gastrointestinal tract and immune system. Concerning protein expression, the kinase is markedly present in almost all human tissues with the exception of adipose and soft tissue and the highest levels in Liver & gallbladder and Kidney & urinary bladder (Protein Atlas).

While mutations of DYRK2 are not often found in cancer cells (the majority of them are missense mutations, with the exception of S471X, a nonsense mutation that lacks kinase activity (Nihira & Yoshida, 2015), a deregulated expression was described by several groups. Further research to elucidate DYRK2 expression in human tumors is needed as different observations have been reported. It has been observed a high expression of DYRK2 in lymphatic system, in muscle-rich tissues, such as skeletal muscle and heart, and in neuroblastoma, esophagus and lung tumors (Miller *et al.*, 2003; Park *et al.*, 2002; Varjosalo *et al.*, 2008). On the other hand, it has been described a reduced or abolished DYRK2 expression in multiple human tumor tissues, which correlates with a shorter survival in the case of lung cancer (Yamashita *et al.*,

2009) and invasiveness in the case of human breast cancer (Taira *et al.*, 2012). Likewise, recent published data correlates low DYRK2 expression with breast cancer recurrence (Enomoto *et al.*, 2014), as well as poor prognosis in colorectal cancer (Yan *et al.*, 2016) and hepatocarcinoma (Zhang *et al.*, 2016). Moreover, DYRK2 has been also proposed as a suppressor and potential prognostic marker for bladder cancer (Nomura *et al.*, 2015) and liver metastasis of colorectal cancer (Ito *et al.*, 2017) and as predictive marker for clinical responses to everolimus in hormone receptor-positive breast cancer (Mimoto *et al.*, 2017).

3.3.4. DYRK2 regulation

DYRK2 is controlled under normal conditions by Mdm2, resulting in constitutive ubiquitination and proteasome degradation. Afterwards DNA damage, ATM phosphorylates DYRK2 on Thr33 and Ser369, which enable DYRK2 to escape from degradation by being dissociated from Mdm2 and to induce its kinase activity toward p53 at Ser46 in the nucleus. Thus, ATM augments nuclear stabilization of DYRK2 by inhibiting MDM2 in the apoptotic response to DNA damage (Taira *et al.*, 2010). Furthermore, DYRK2 is controlled by MAP3K10 affecting its activity in the Hedgehog signaling pathway. MAP3K10 directly phosphorylates DYRK2 on Ser376 and Thr308. It is worth pointing out the closeness of the latter target residue to tyrosine 309, a critical activating autophosphorylation site found in all DYRK kinases (Varjosalo *et al.*, 2008). Under hypoxic conditions, SIAH2 mediates DYRK2 polyubiquitination and subsequent proteasome degradation, which dampens the ability of DYRK2 to fuel the hypoxic response and alters the p53-dependent apoptosis in response to DNA damage (Perez *et al.*, 2012). Moreover, the centrosomal protein Cep78 controls the scaffold function of DYRK2 due to its binding to the EDD-DYRK2-DDB1^{VprBP} E3 ligase complex. This interaction inhibits the complex activity, affecting centriole length and cilia assembly, and therefore, centrosome homeostasis (Hossain *et al.*, 2017). Finally, it has been recently reported how miRNAs are also capable to regulate DYRK2 expression. DYRK2 was negatively regulated by miR-622 via a specific targeted binding site within the 3'UTR. Interestingly, decrease of miR-622 expression suppresses migration and invasion by targeting regulation of DYRK2 in colorectal cancer cells (Wang *et al.*, 2017).

Besides the biochemical regulation of DYRK2, there exists two natural compound, harmine from *Banisteriopsis caapi* and leucettine from the marine sponge alkaloid leucettamine B, with the ability to inhibit DYRK kinase activity (Gockler *et al.*, 2009; Tahtouh *et al.*, 2012).

3.3.5. CDC25A

Cell division cycle 25A (CDC25A) is a member of the CDC25 family of highly conserved dual-specificity phosphatases (dephosphorylates both tyrosine (Y) and threonine (T) residues). They are key regulators of normal cell division and mediators of the checkpoint response to DNA damage. During normal cell division they play a fundamental role in transitions between cell cycle phases, via dephosphorylation and consequent activation of Cyclin-dependent kinases (CDK) / Cyclin complexes. To date, CDK/Cyclin complexes are the only known substrates for CDC25 phosphatases (Boutros *et al.*, 2007). The three isoforms of the family, CDC25A, CDC25B and CDC25C, are essential for the proper execution of the cell cycle, although it has been shown that CDC25A alone is sufficient for initiation of each step in the cell cycle (Ferguson *et al.*, 2005).

The human CDC25A protein, encoded by Cdc25A gene located on chromosome 3p21.31, has 524 amino acid residues, with two distinct regions: a C-terminal catalytic domain and an N-terminal regulatory domain that contains phosphorylation sites involved in regulating protein stability and protein-protein interaction. Some of these sites constitute functional regulatory domains, such as DSG and KEN motifs. Besides, the regulatory domain also presents nuclear localization sequence (NLS) and nuclear exportation sequence (NES), which determine the subcellular location of CDC25A (Shen & Huang, 2012) (Figure 7A). Two isoforms of human CDC25A have been reported of which transcript variant 1 contains 121 nucleotides more (from 793 to 914) than transcript variant 2.

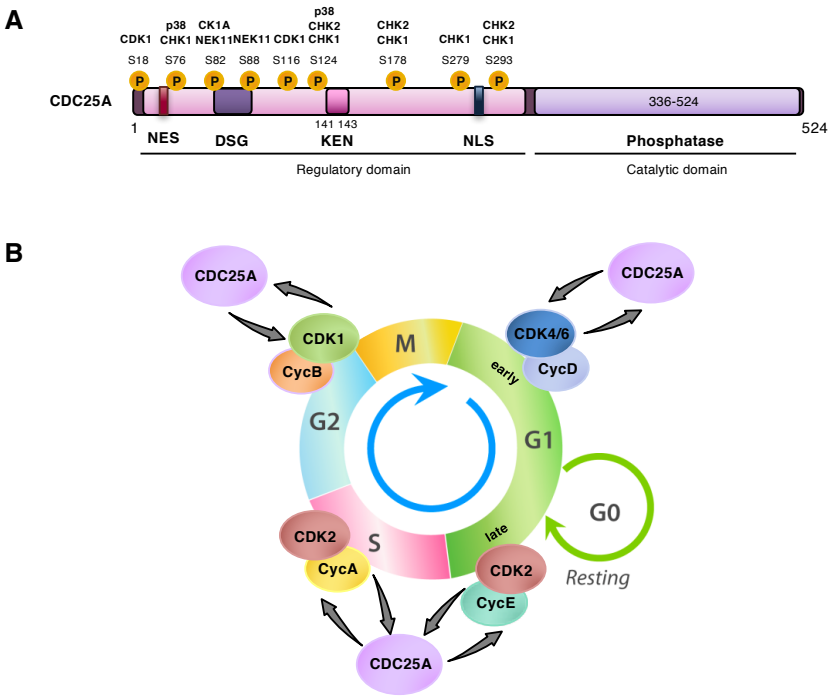

Figure 7. Scheme of CDC25A structure and function. A. Scheme of CDC25A protein. Regulatory and catalytic domains are represented, including DSG and KEN regulatory motifs and phosphorylated residues involved in the regulation of the phosphatase. **B.** Role of CDC25A in cell cycle regulation, showing the feedback loops generated with CDK/Cyclin complexes.

In mammalian cells, CDK/Cyclin complexes are kept inactive by phosphorylation on specific residues. Activation of these complexes by dephosphorylation is needed to lead cells to go into cell cycle progression, task that is mediated by CDC25 phosphatases. While CDC25B and CDC25C promote G2/M progression by primarily dephosphorylating CDK1 at T14/Y15, CDC25A plays a more extensive role in assisting both G1/S and G2/M progression. CDC25A is an unstable protein whose cellular levels are regulated by periodic synthesis and by ubiquitin mediated proteolysis. In late G1 phase, CDC25A RNA and protein levels increase as cells are stimulated to enter the cycle from quiescence, as a result of E2F-1- and c-Myc-mediated transcriptional activation (Galaktionov *et al.*, 1996; Vigo *et al.*, 1999). CDC25A mainly activates the CDK2/Cyclin E and CDK2/Cyclin A complexes during the G1–S transition, by removing CDK2 T14 and Y15 inhibitory phosphorylations. In turn,

CDK2 creates an auto-amplification loop further phosphorylating and activating CDC25A, what contributes to S phase progression by recruiting the essential replication factor CDC45 and DNA polymerase on replication origins. Moreover, during G1-S transition CDC25A plays another role through the activation of CyclinD-CDK4/6 complexes, by removing CDK4 Y17 and CDK6 Y24 respectively (Iavarone & Massague, 1997; Terada *et al.*, 1995). Likewise, CDK4/6-CyclinD complexes mediate the phosphorylation of CDC25A on S40 during G1, decreasing its stability in a ßTrCP-dependent manner (Dozier *et al.*, 2017). Furthermore, CDC25A also has a remarkable role in the G2/M transition, by activating CDK1/Cyclin B complexes through the revoking of its phosphate groups on T14 and Y15. Once active, CDK1/Cyclin B complexes contribute to initiate chromosome condensation, what lastly allow cells moving from interphase to mitosis. As it occurs with previous CDK/Cyclin complexes mentioned above, CDK1/Cyclin B generates a feedback loop (through CDC25A phosphorylation on S18 and 116) that stabilizes the phosphatase, allowing control of the G2-M transition (Boutros *et al.*, 2007; Busino *et al.*, 2004) (Figure 7B).

Due to its major function, CDC25A regulation results essential for the proper cell division. To ensure timely progression through the various phases of the cell cycle, the phosphatase expression and activity is tightly regulated by many mechanisms, including alternative exon splicing, phosphorylation–dephosphorylation cycles, interactions with partners such as 14-3-3 proteins, intracellular localization and cell-cycle controlled degradation. CDC25A is constantly turned over in cycling cell. It first appears in late G1, and until mitosis, continuous de novo CDC25A synthesis is counterbalanced by its degradation through the ubiquitin–proteasome pathway (Busino *et al.*, 2004). Two ubiquitin ligase complexes have been described to mediate CDC25A ubiquitylation during cell cycle: APC/CCdh1 and $SCF^{\beta TCRP}$. Each complex act at distinct stages of the cell cycle (at the end of mitosis and in S and G2 phases, respectively) and specific recognition motifs in CDC25A are required for the interaction. Specifically, $SCF^{\beta TCRP}$ binding is dependent of phosphorylation of serine residues before and/or within the DSG motif, while interaction with APC/CCdh1 is dependent upon a KEN motif without apparent requirement for post-translational modifications (Busino *et al.*, 2003; Donzelli *et al.*, 2002). The phosphorylation of the N-terminal regulatory domains is

performed by several kinases, including the CDK/Cyclin complexes themselves, CHK1, CHK2 and p38. CHK1 controls the proper timing of mitotic onset by phosphorylating CDC25A and thereby creating a 14-3-3 binding site to prevent its interaction with CDK1/Cyclin B. CDC25A is predominantly expressed in G1 and stabilized in mitosis through CDK1/CyclinB-mediated phosphorylation (Ser18 and 116). At the end of mitosis, CDC25A levels rapidly decrease owing to its degradation, mediated by APC/CCdh1-dependent ubiquitylation. In S and G2 phases, CDC25A is targeted for proteasome-mediated degradation by $SCF^{\beta TCRP}$, requiring previous phosphorylation of serine clusters by CHK1 (Ser76, 124, 178, 279, 293), CHK2 (Ser124, 178, 279), p38 (Ser76, 124), CK1A (Ser82), NEK11 (Ser82, 88) and another possible as yet unknown kinase (Boutros *et al.*, 2007; Busino *et al.*, 2004; Honaker & Piwnica-Worms, 2010; Melixetian *et al.*, 2009). Moreover, a recent study identified Cul4B-$DDB1^{DCAF8}$ as a new CDC25A E3 ligase, whose function is dependent of previous deacetylation at lysine 150 by HDAC3 (Wu *et al.*, 2016).

As mentioned above, CDC25A is a key target of the checkpoint machinery activated in response to DNA damage. It is specifically and rapidly degraded by the proteasome via phosphorylation of kinases of the activated ATM/ATR–CHK1/2 pathway, in order to stop cell cycle progression meanwhile DNA damaged is repaired, preventing cells with chromosomal abnormalities from progressing through cell division (Reinhardt & Yaffe, 2009). The basal turnover of CDC25A controlled by CHK1 phosphorylation (residues Ser124, 178, 279, 293) is accelerated in response to UV light or IR treatment, when an increase in phosphorylation at Ser124 is detected. This leads to its ubiquitin-mediated destruction or loss of phosphatase activity and induces a G2 arrest. IR-induced activation of CHK2 cooperates with CHK1 in phosphorylating CDC25A on serine 124, 178 and 293. It has also been demonstrated that CHK1 and p38 dependent Ser76 phosphorylation of CDC25A mediates its stabilization in response to UV-induced DNA damage (Boutros *et al.*, 2006; Busino *et al.*, 2004).

CDC25A overexpression, which can be caused by stabilization of the protein, accelerates the G1/S and G2/M transitions, leading to genomic instability and promoting tumorigenesis. This deregulated expression may be due to anomalous E2F1/c-Myc

E2F1/c-Myc transcriptional activity or alternatively to deregulations at different levels of the degradation pathway of CDC25A (disrupted CHK1/CHK2 activities, mutations in the ubiquitin ligases that mediate the degradation or even mutations in specific consensus sites (e.g. DSG motif) of the phosphatase itself) (Busino *et al.*, 2004). CDC25A has been reported to be overexpressed (both at the mRNA and protein levels) in primary tissue samples from various human cancers, including breast, colorectal, lung pancreas, hepatocellular, thyroid and non-Hodgkin lymphoma among others, being highly associated with the malignancy and poor prognosis in cancer patients. For instance, CDC25A overexpression has been associated with shorter disease-free survival in breast, esophageal and hepatocellular carcinomas (Boutros *et al.*, 2007; Kristjansdottir & Rudolph, 2004). Therefore, CDC25A inhibition is considered as a potential therapeutic target against cancer. However, most of the inhibitors described to date show cross-reaction with the three CDC25 isoforms, lacking specificity over CDC25A (Brenner *et al.*, 2014).

Aims

Cancer is an evolutionary disease fueled by genomic instability due to alterations of DNA Damage Response pathway, among others. Although there is a great knowledge of the signaling networks that comprise this complex pathway, further research is needed to elucidate new molecular mechanisms whereby DNA-damage cells tip the balance to apoptosis or tumorigenesis. In this sense, it is relevant to study how kinases involved in the DDR pathway, such as CHK2 and DYRK2, exerts its function during tumour development and progression, through the identification of new substrates that take an active part in these processes. Thereby, our understanding of the tumorigenic process will be improved, opening a road to the development of new therapeutic strategies against cancer.

The aims of this search project have been the following:

1. Identify and analyse new kinases (CHK2 and DYRK2) substrates involved in the DNA Damage Response pathway.

2. Study the biochemical consequences of CHK2 and DYRK2 regulation over their identified substrates, with a special focus under genotoxic stress conditions.

3. Investigate the functional implications of CHK2 and DYRK2 regulation over their identified substrates in carcinogenesis, through analyses in cancer cells and tissues.

Material and Methods

5.1. *Cell culture and hypoxia*

HEK-293T (Human embryonic kidney cells), HeLa (human cervical cancer cells), U2OS (human osteosarcoma cell line), A549 (human lung cancer cells) wild type (wt)/cisplatin resistant (CPR), control MEFs (mouse embryonic fibroblasts) and Siah1a$^{-/-}$ /Siah2$^{-/-}$ MEF cells were maintained in DMEM. H727 (human lung cancer cells) and MOR (human lung cancer cells) wt/CPR cells were maintained in RPMI. Both DMEM and RPMI were supplemented with 10% FCS, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2. Cell lines are routinely tested to be free of mycoplasma and cross contamination. Cell lines validation was performed by a multiplex PCR with Geneprint10 System (Promega, Madison, WI, U.S.A.). HEK-293T, HeLa, U2OS, control MEFs and H727 cells were obtained from ATCC. A549 wt/CPR cells and Epo-Luc plasmid were a gift from Dr. M. L. Schmitz (University of Giessen, Germany). MOR wt/CPR cells were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Siah1a$^{-/-}$ /Siah2$^{-/-}$ MEF cells were obtained from Dr. David Bowtell and Dr. Andreas Möller. HIPK2$^{-/-}$ HEK-293T cells were kindly provided by Dr. Laureano de la Vega (University of Dundee, Scotland, UK). Hypoxia was induced by cultivation of cells in a New Brunswick Galaxy 48 R incubator (Eppendorf, Hamburg, Germany) at defined O_2 concentrations.

5.2. *Reagents*

MG-132 was purchased from Enzo Life Science (Lausen, Switzerland), Caffeine (84677) was from Fluka (Bucharest, Romania), Calyculin A (sc-24000) from Santa Cruz (Santa Cruz, CA, U.S.A.) and the rest of the reagents were from Sigma-Aldrich: Etoposide (E1383), Cisplatin (P4394), 5-Fluorouracil (5-FU) (F6627), Cicloheximide (C7698), Thymidine (T9250) and Adriamycin/Doxorubicin hydrochloride (44583). Scramble control oligonucleotide siRNA non-targeting pool (D-001810-10-20) and the siGENOME SMARTpool against SIAH2 (M-006561-02), ON-TARGET plus SMARTpool against CHK2 (L-003256-00) and ON-TARGET plus SMARTpool against DYRK2 (L-004730-00-0010) were purchased from Dharmacon (Waltham, MA, U.S.A.). The effect of siRNA on protein expression was assessed after 48 h of transfection for CHK2 and SIAH2 and after 72 h for DYRK2. E1, E2-UbcH5c, Ubiquitin and CDC25A

(ab90763) human recombinant proteins were purchased from Abcam (Cambrigde, UK). DYRK2 (14-669) recombinant protein was from Merck (Massachusetts, U.S.A.).

5.3. *Transfection and plasmids*

Transient transfections were performed with Rotifect (CarlRoth, Karlsruhe, Germany) according to manufacturer instructions and harvested 48 h after transfection. DNA amounts in each transfection were kept constant upon addition of empty expression vector.

Flag-CHK2, KD (Kinase Dead) and T68A, Myc-CHK2, KD were a gift from Dr. H. Piwnica-Worms (Washington University School of Medicine, U.S.A.). GST-CHK2 plasmid was a gift from Dr. Stephen J. Elledge (Harvard Medical School, U.S.A.). HA-CHK2, KD, GST-CHK2-ΔSCD and ΔFHA plasmids were kindly provided by Dr. David F. Stern (Yale University School of Medicine). HA-CHK2 S456A was provided by Dr. Carol Prives (Department of Biological Sciences, Columbia University, New York). CHK2-GFP was provided by Dr. L. Zannini (Istituto Nazionale dei Tumori, Milan, Italy). Myc-CHK2 ΔSCD and ΔFHA were kindly provided by Dr. S-Y Shieh (Institute of Biomedical Science, Taiwan). Flag-CHK2 S19A was a gift from Dr. Domenico Delia (Istituto Nazionale dei Tumori, Milan, Italy). Flag-PHD3 was a gift from Dr. Frank S. Lee (Pennsylvania School of Medicine). Flag-SIAH2 and RM (Ring Mutant, H98A/C101A), Flag-SIAH2-3A (triple mutant) and Flag-SIAH2-3D (phosphomimic), HA-SIAH2 and RM, His-Ubiquitin and Flag-HIPK2 and KD were described previously (Calzado et al, 2009). Flag-DYRK2 and KD (kinase dead, K251R), GFP-DYRK2 and KD and HA-DYRK1A/DYRK3/DYRK4 were gifts from Dr. Susana de la Luna (Centre for genomic regulation, Barcelona, Spain). Myc-DYRK2 and KD were cloned by PCR (from Flag-DYRK2 and KD) to pCMV-Tag3B vectors. GFP-DYRK1B was kindly provided by Dr. Walter Becker (Rwth Aachen University, Germany). Flag-CDC25A was kindly provided by Dr. Peter Stambrook (University of Cincinnati, Ohio, U.S.A.). Flag-CDC25A-S88A, Flag-CDC25A-ΔKEN and Flag-CDC25A-DM were obtained by performing directed mutagenesis from Flag-CDC25A plasmid using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, California, U.S.A.) according to manufacturer instructions. All the constructs generated were confirmed by DNA sequencing.

5.4. *BEAS-2B squamous-cell differentiation model*

BEAS-2B cells were purchased from SIGMA (SIGMA; European Collection of Cell Cultures, Salisbury, UK. 95102433) and maintained in serum free LHC-9 medium (Life Technologies, Carlsbad, CA, U.S.A.) at 37 °C in a humidified atmosphere containing 5% CO_2. Squamous differentiation was acquired after culturing in LHC-9 medium enriched with 10% FBS during 7 days.

5.5. *Cell synchronization*

HeLa cells were synchronized in G1 phase of the cell cycle by Double Thymidine Block. For first blockade 2 mM thymidine was added to the cells during 18 h. Then cells were washed with 1X PBS and incubated with fresh media for 9 h. Next, 2 mM thymidine was added again for second blockade during 15 h. Finally, cells were released by washing with 1X PBS and adding fresh DMEM and harvested after 0 h (G1 fraction), 3.5 h (S fraction), 6 h (G2 fraction) and 10 h (M fraction). Checking of the cellular fractions was performed by flow cytometry (FACS analyses).

5.6. *FACS analyses*

For cell cycle analysis, cells were fixed in 70% cold ethanol at -20 ºC overnight. After that cells were washed with PBS once and then stained with 1 mg/mL propidium iodide (PI) and treated with RNase A (50 U/mL) for 2 h at 37 ºC in darkness. For apoptosis studies, cells were harvested and washed in cold PBS and then resuspended in binding buffer consisting of 10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$ pH 7.4. Cells were stained with Annexin V, Alexa Fluor 488 conjugate (Molecular Probes by Life Technologies, Carlsbad, CA, U.S.A.) and propidium iodide. Cell cycle distribution and apoptosis were determined by BD FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) using BD FACSDiva™ software.

5.7. *Cell lysis and Western Blotting*

Soluble fractions were obtained after lysis of cells in NP-40 buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) NP-40, 10% (v/v) glycerol, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (10 µg/ml), leupeptine (10 µg/ml), pepstatin (1 µg/ml) and 1 mM PMSF saturated]. After centrifugation the supernatants were mixed with SDS sample buffer (50mM Tris-HCl pH6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and boiled at 95 °C. Proteins were resolved on SDS-PAGE gels and blotted to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer. After blocking with non fat milk or BSA in TBST buffer, primary antibodies were added. Appropriate secondary antibodies coupled to horseradish peroxidase were detected by an enhanced chemiluminescence system (USB).

5.8. *Antibodies*

Mouse monoclonal antibodies against Flag (M2) and anti-β-actin (AC-74) were purchased from Sigma-Aldrich, anti-HA epitope (3F10), anti-GFP (11814460001) and anti-myc (9E10) from Roche Molecular Biochemicals (Mannheim, Germany), anti-p53 phospho Ser46 (2521), anti-ubiquitin (P4D1), anti-CHK2 Thr68 [(C13C1)-2197], anti-CDC25C Ser216 (9528), anti-CHK2 Ser516 (2669) and anti-p53 Ser20 (9287) from Cell Signaling Technology (Danvers, MA, U.S.A.), anti-DYRK2 (sc-66867), anti-SIAH2 (sc-5507) and anti-p53 (sc-126) from Santa Cruz, anti-CDC25A (MA5-13794) from Thermo Fisher Scientific (Waltham, MA U.S.A.), anti-phospho-S/T-Pro (05-368) from Merck (Massachusetts, U.S.A.). Rabbit polyclonal antibodies recognizing phosphorylated threonine 26 and serines 28 and 68 of SIAH2 were raised by NeoMPS (San Diego, California, U.S.A.). Rabbit polyclonal antibodies recognizing phosphorylated threonine 119 of SIAH2 were raised by Abyntek (Vizcaya, Spain). Alexa Fluor 647 goat anti-mouse IgG1 (A21235) antibody was from Life technologies (Carlsbad, CA, U.S.A.). Secondary HRP-coupled antibodies were obtained from Jackson ImmunoResearch Laboratories (Baltimore, U.S.A.).

5.9. *Immunoprecipitation*

Cells were first washed in PBS and collected by centrifugation. The cell pellet was lysed in IP buffer [(50 mM Hepes pH 7.5, 50 mM NaCl and 1% Triton X-100) supplemented with 5 mM EGTA, 20 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF and 10 µg/ml of leupeptine, aprotinin and pepstatin]. Cell lysates were incubated with 1 µg of the indicated antibodies for 6 h at 4 ºC. Antibodies were then isolated with 25 µl of protein A/G Sepharose (Santa Cruz). Imnunoprecipitated proteins were then washed five times in IP buffer and eluted in 1.5X SDS sample buffer. Samples were immunoblotted as described before.

5.10. *Immunofluorescence*

Cells were grown on coverslips and 48 h after transfection fixed with 3.7% of pre-warmed paraphormaldehyde/PBS for 10 minutes. Cells were then permeabilized with 0.1% Triton X-100/PBS for 15 minutes blocked with 3% BSA/PBS and incubated overnight with primary antibodies. After being washed with PBS and incubated for 45 min with the secondary antibody they were mounted on glass slides with mounting medium with DAPI (Vectashield). Fluorescence images were captured using confocal laser scanning microscope LSM 5 EXCITER (Carl Zeiss MicroImaging GmbH) using a 40X/1.30 oil objective (EC Plan-Neofluar) and ZEN 2008 software (Carl Zeiss MicroImaging GmbH). Images were analysed using the ImageJ v 1.45 software (http://rsbweb.nih.gov/ij/). The Costes' approach was employed for co-localization analysis between green (Alexa 488) and red (Alexa 647) images to determinate the Pearson's correlation coefficient (R). SIAH2 wild type and phospho mutant versions were visualized on a BD Pathway 855 Bioimager in a non-confocal mode.

5.11. *Immunohistochemical analysis*

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded human lung tumors. Staining of cancer cells was scored and compared to peripheral normal lung tissue. Five-micrometer sections from tumor samples were prepared for tissue slides. Sections were deparaffinised in xylene, and rehydrated in a

graded ethanol series and distilled water. Staining with hematoxylin–eosin (H & E) and the rabbit polyclonal anti-DYRK2 antibody (AP7534a; Abgent, San Diego, California, U.S.A.) at 1:50 dilution was performed overnight at 4 °C. Antigen retrieval for sections to be stained with the polyclonal anti-DYRK2 antibody was performed by incubating the slides in 1 mM EDTA pH 8.0 in a 98 °C–100 °C steamer for 30 minutes. DYRK2 IHC staining was evaluated as follows: 0, no staining or faint cytoplasmic staining in less than 10% of tumor cells; 1+, faint cytoplasmic staining inmore tan 10% of tumor cells; 2+, weak or moderate cytoplasmic staining inmore than 10% of tumor cells; 3+, more than 10% of strong cytoplasmic staining. 0 or 1+ staining intensity was considered as DYRK2 negative, whereas 2+ or 3+ was considered as DYRK2 positive.

5.12. *Enrichment of His-Tagged Proteins*

Cells were collected in PBS and pellets were dissolved in lysis buffer [50 mM Tris-HCl buffer pH 8.0 containing 8M urea, 50 mM Na_2HPO_4, 300 mM NaCl, 0.5% (v/v) NP-40, 10 mM imidazole and 1 mM β-mercaptoethanol]. Samples were sonified and cell debris was removed by centrifugation. His-ubiquitin tagged proteins were isolated with 40 µl of equilibrated Ni-NTA resin (Qiagen) for 4 h at room temperature. After centrifugation precipitates were washed once with lysis buffer, once with wash buffer 1 (10 mM Tris HCl, pH 8.0 containing 8M Urea, 100 mM Na_2HPO_4, 10 mM imidazole and 1 mM β-Mercaptoethanol) and twice with Wash buffer 2 (the same composition of wash buffer 1 but at pH 6.3). Proteins were eluted in SDS sample buffer containing 200 mM imidazole and analyzed by immunoblotting.

5.13. *GST pull-down assay*

Recombinant GST-SIAH2 proteins (wt, ΔC and ΔN) and GST-CHK2 (wt, ΔSCD and ΔFHA) were produced in *Escherichia coli* BL21 cells and purified with Glutathione-Sepharose 4B (GE Healthcare) according to standard protocols. Soluble fusion proteins (10 µg) were incubated at 4 ºC overnight with 30 µl of glutathione-sepharose beads. Sepharose beads were then washed with IP buffer and resuspended in SDS sample buffer for further inmunoblot analysis.

5.14. *In vitro ubiquitination analysis*

Recombinant GST-CHK2 was incubated or not with different combinations of E1, E2-UbcH5c, E3 (GST-SIAH2) and ubiquitin human recombinant proteins in ubiquitylation buffer (50 mM Tris, 5 mM MgCl2, 0.1% Tween-20, 1 mM DTT) and ATP 2mM. After 60 min of incubation at 37 ºC reactions were stopped adding SDS sample buffer and samples were subjected to Western Blot analyses.

5.15. *In vitro phosphorylation analysis*

GST-SIAH2 fusion protein was incubated with GST-CHK2 protein in kinase buffer (20 mM Hepes pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mM ATP). After 60 min of incubation at 30 ºC reactions were stopped adding SDS sample buffer. Samples were then immunoblotted with anti-PhosphoSer28-SIAH2 and anti-CHK2 antibodies. Similarly, commercial CDC25A recombinant protein was incubated with DYRK2 recombinant protein in kinase buffer and proceeded as mentioned before. Samples were immunoblotted with anti-Phospho-S/T-Pro, anti-CDC25A and anti-DYRK2 antibodies.

5.16. *γ-phosphatase assay*

Cells were lysed in NP-40 buffer without phosphatase inhibitors. Then samples were treated with γ-phosphatase following manufacturer's instructions (New England Biolabs). Reactions were stopped with SDS buffer and samples inmunoblottted.

5.17. *Peptide array-binding assay*

Overlapping dodecapeptides of SIAH2 protein were automated spotted on cellulose membranes by using Fmoc-protection chemistry. Membrane was blocked overnight in non fat milk in TBS buffer and incubated during 6 hours with 60 nmol of recombinant GST-CHK2 protein or GST protein for control. Then primary anti-CHK2 antibody was added and mouse secondary antibodies coupled to horseradish peroxidase were revealed by enhanced chemiluminescence system (USB) on autoradiographic films.

5.18. *mRNA extraction and qPCR*

Cells were harvested and washed twice with PBS. The cell pellet was lysed and total RNA was extracted using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). RNA integrity was checked on an agarose gel (Bio-Rad Universal Hood II). mRNA retrotranscription was performed with the iScript cDNA Synthesis kit (Bio-Rad, Madrid, Spain). Real-time PCR was employed with iQ™ SYBR® Green Supermix (Bio-Rad) in an iCYCLER detection system (Bio-Rad). Amplification efficiencies were validated and normalized against β-actin, and fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used:

CHK2-F: 5'-CGGATGTTGAGGCTCAGCA-3'
CHK2-R: 5'-TATGCCCTGGGACTGTGAGG-3'
CDC25A-F: 5'-GCACTCGGTCAGTGTTGAAG-3'
CDC25A-R: 5'-CATGGGCCTTCTCTGGATTA-3'
HPRT-F: 5'-AATTATGGACAGGACTGAACGTCTTGCT-3'
HPRT-R: 5'-TCCAGCAGGTCAGCAAAGAATTTATAGC-3'

5.19. *Luciferase reporter assays*

Cells were collected in PBS and lysed in luciferase buffer (25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol). Luciferase assay was performed using Luciferase Assay Reagent (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions and luciferase activity was measured in a luminometer Autolumat LB9510 (EG&G Berthold, U.S.A.). Luciferase activity in different samples was normalized with protein concentration.

5.20. *Cell viability assays*

Cell viability was measured by MTT assay. MTT reagent (5 mg/ml, Sigma-Aldrich) was added to cells and incubated for 3 h at 37 °C in darkness. The formazan crystals were solubilized by the addition of 100 µL DMSO and the absorbance at 550 nm was measured using a Multifunction Microplate Reader (TECAN GENios Pro, Switzerland). All determinations were carried out in triplicate.

5.21. *Statistical analysis*

Data are expressed as mean ± SD. Differences were analyzed by Student's t test. Statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad, San Diego, CA, U.S.A.). $P<0.05$ was considered significant. Images were analyzed and quantified using the ImageJ v1.45 software.

Results

6.1. CHK2 stability is regulated by the E3 ubiquitin ligase SIAH2

6.1.1. SIAH2 interacts and colocalizes with CHK2

In order to investigate the mechanisms responsible for maintaining the steady-levels of CHK2, we tested a set of different E3 ligases for their ability to interact with CHK2 (data not shown). As SIAH2 showed positive results in our screening, we decided to focus on this protein in detail. We first co-expressed Flag-CHK2 alone or in the presence of hemagglutinin epitope (HA)-tagged SIAH2 in HEK-293T cells, and performed coimmunoprecipitation assays. Flag-CHK2 coimmunoprecipitated efficiently with HA-SIAH2 (Figure 8A). Similar results were obtained the other way round (Figure 8B). Similarly, immunoprecipitation of endogenous CHK2 pulled down SIAH2 (Figure 8C).

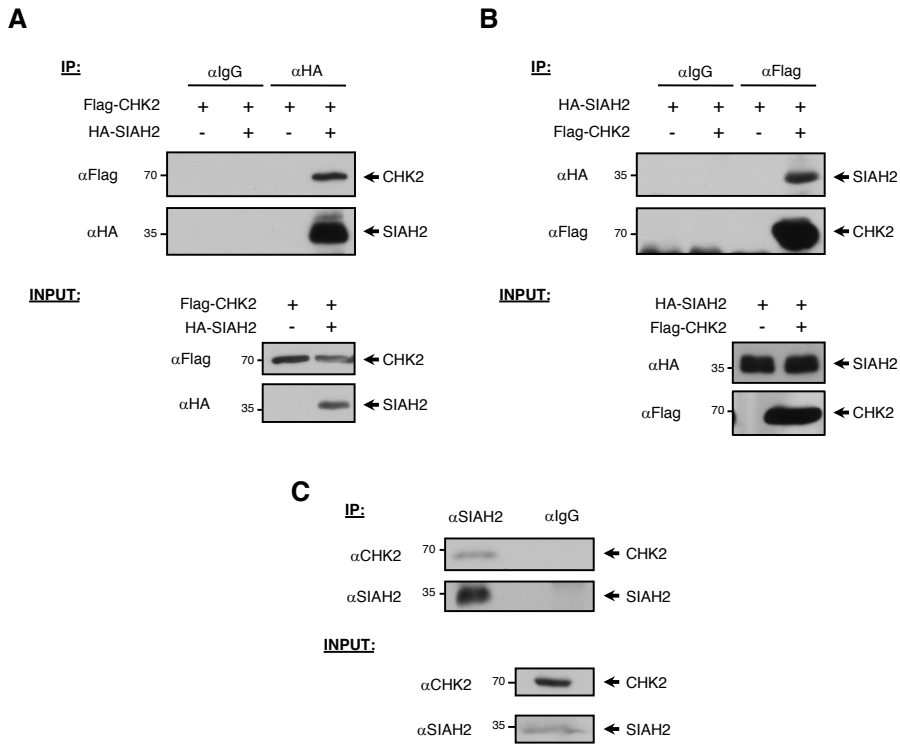

Figure 8. CHK2 interacts with SIAH2. (A) HEK-293T cells were transfected with expression plasmids encoding HA-SIAH2 and Flag-CHK2 as indicated, and after 36 h the proteasome inhibitor MG-132 (10 μM) was added for another 12 h to avoid SIAH2 autodegradation. Cells were lysed and subjected to immunoprecipitation (IP) using anti-HA antibody. After elution, Flag-

CHK2 protein was detected by western blotting (WB). A small fraction (5%) of the lysate was tested for the occurrence of the indicated proteins by immunoblot (INPUT). The positions and molecular weights (in kDa) are indicated. We show a representative blot of three independent experiments. **(B)** The experimental settings were similar to (A) with the exception that the inmunoprecipitation was performed using anti-Flag antibody for CHK2. We show a representative blot of three independent experiments. **(C)** HeLa cells were lysed and a fraction subjected to IP with anti-SIAH2 antibody or Ig control. The precipitates were subjected to WB analysis with anti-CHK2 or anti-SIAH2 antibodies. We show a representative blot of three independent experiments.

Then, we analyzed the subcellular localization of both proteins and the effect of DNA damage by ETP treatment. CHK2 and SIAH2 proteins mainly colocalize in the nucleus and no changes were observed in the cells stimulated with ETP (Figure 9).

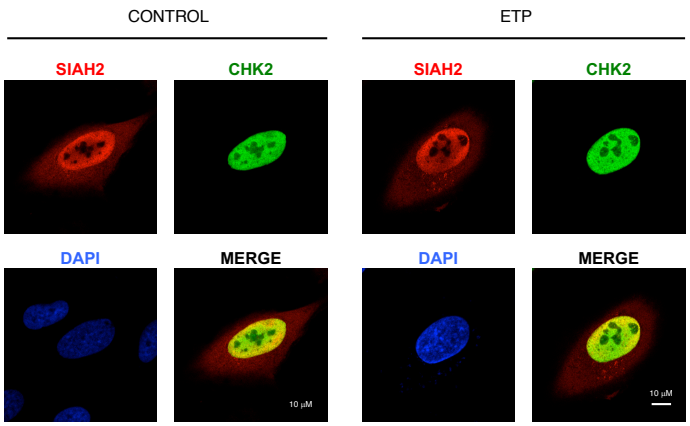

Figure 9. CHK2 colocalizes with SIAH2. U2OS cells were transfected with Flag-SIAH2 RM (RING Mutant) and CHK2-GFP and analyzed for the localization of both proteins by immunofluorescence. Nuclear DNA was stained with DAPI. Overlapping localization in merged pictures is shown in yellow. We show a representative picture. Correlation analysis revealed a high degree of colocalization with a value of 0.59 ± 0.06 for Pearson's coefficient. No significant changes were observed in the cells stimulated with ETP (0.61 ± 0.07).

Next, we wanted to study the effect of DNA damage on CHK2-SIAH2 interaction. Coimmunoprecipitation experiments showed that the interaction between these two proteins was markedly reduced under ETP (Figure 10A and 10B), cisplatin or 5-fluorouracil (5-FU) stimulation (Figure 10B). All these data support the existence of a CHK2-SIAH2 complex, which can be partially disrupted in response to DNA damage without impairing its subcellular colocalization.

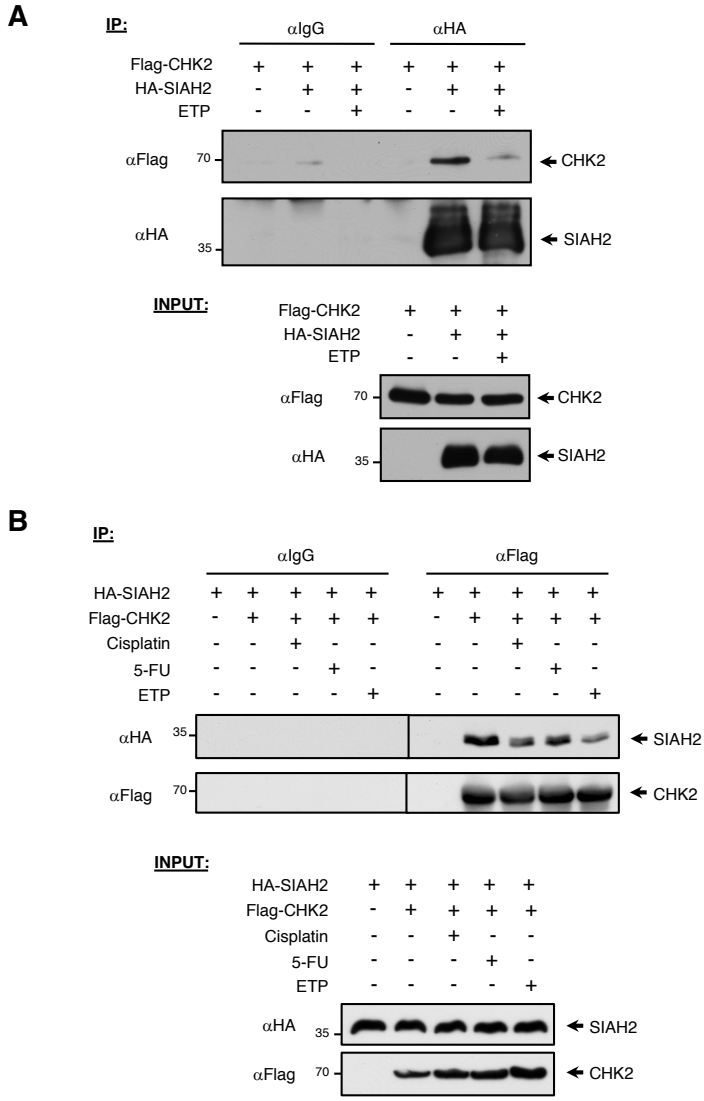

Figure 10. DNA damage effect on CHK2-SIAH2 interaction. (A) HEK-293T cells were transfected with the indicated plasmids, and after 36h stimulated with ETP (10 μM) and MG-132 for another 12 h. Cells were lysed, subjected to immunoprecipitation (IP) using anti-HA antibody, and the different proteins detected by WB. A small fraction (5%) of the lysate was tested by immunoblot for the occurrence of the indicated proteins (INPUT). We show a representative blot of four independent experiments. **(B)** HEK-293T cells were transfected with the indicated plasmids, and after 36 h stimulated with ETP (10 μM), cisplatin (5 μM) or 5-FU (0.5 μg/ml) as indicated, and MG-132 for another 12 h to avoid SIAH2 autodegradation. Cells were lysed, subjected to IP using anti-Flag antibody, and, after elution, detected by WB. A small fraction (5%) of the lysate was tested for the occurrence of the indicated proteins by immunoblot (INPUT).

6.1.2. CHK2 shows a direct interaction with SIAH2

To study whether CHK2 and SIAH2 directly interact and to characterize the responsible domains of this interaction, we performed GST-pulldown assays. Flag-CHK2 was brought down by full-length SIAH2 and also by two deletion mutants (N- and C-terminus) (Figure 11A). Similarly, Flag-SIAH2 was captured by full-length CHK2 and, to a lesser extent, by FHA or SCD domain-deleted mutants (Figure 11B).

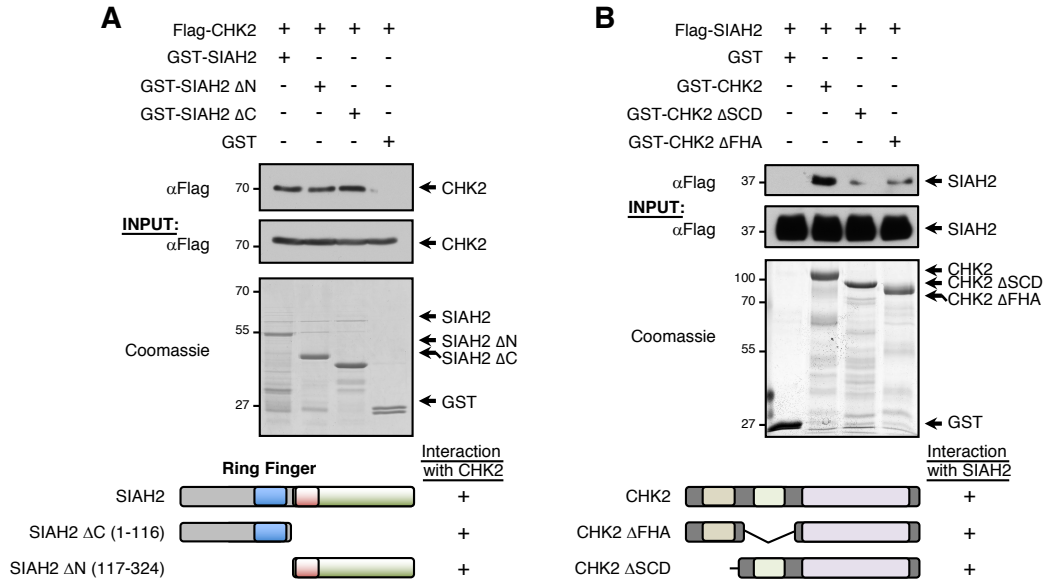

Figure 11. CHK2 shows direct interaction with SIAH2 by GST pull down assays. (A) HEK-293T cells were transfected to express Flag-CHK2, lysed and incubated with GST-SIAH2 or deleted versions. After immunoprecipitation, CHK2 protein was detected by WB with anti-Flag antibody. The lower part shows the Coomassie-stained input material and a schematic representation of SIAH2-WT and its mutants. We show a representative blot of three independent experiments. **(B)** The experimental settings were similar to (A) with the exception that HEK-293T cells were transfected to express Flag-SIAH2, lysed and incubated with GST-CHK2 or deleted versions. We show a representative blot of three independent experiments.

To identify the sites of direct interaction, we performed a peptide array experiment (Figure 12A). Detection of the bound material by antibodies showed that CHK2 binds four distinct domains of SIAH2. Likewise, SIAH2 showed interactions with two distinct sites of CHK2 (Figure 12B). Then, we compared the interaction between

SIAH2 and full-length CHK2 with FHA or SCD domain-deleted mutants by coimmunoprecipitation assay. As shown in Figure 13A, and in agreement with the results obtained in the GST pull-down assays, FHA or SCD domain-deleted mutants were captured to a lesser degree than full-length CHK2 by Flag-SIAH2. Lastly, we decided to compare the interaction between SIAH2 and CHK2 wild-type (wt) or CHK2 kinase dead (KD) mutant by coimmunoprecipitation assays. These experiments revealed that the loss of CHK2 kinase activity does not affect the ability to interact with SIAH2 (Figure 13B). All these results clearly show a direct physical interaction of CHK2 and SIAH2, suggesting the existence of more than one domain responsible for binding in both proteins. Similarly, these data indicate that SIAH2 interacts with CHK2 monomeric and dimeric forms.

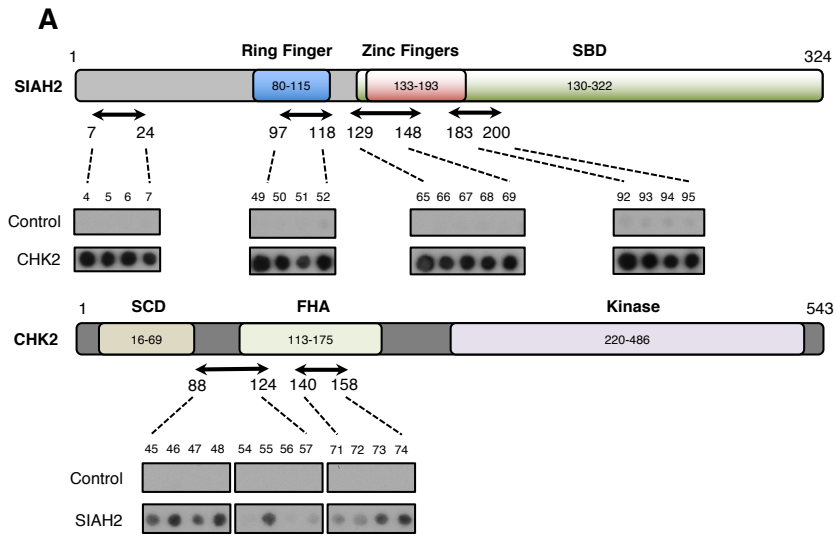

Figure 12. Identification of direct interaction sites in CHK2-SIAH2 complex. (A) Schematic representations of SIAH2 and CHK2 with the regions responsible for interaction. Recombinant GST-CHK2 or GST-SIAH2 proteins (or GST control protein) were incubated with a peptide array library covering the complete sequence of SIAH2 or CHK2 respectively, and bound proteins were revealed by immunoblot. CHK2 binds to four distinct domains of SIAH2, while SIAH2 interacts with two domains in the lower part of CHK2.

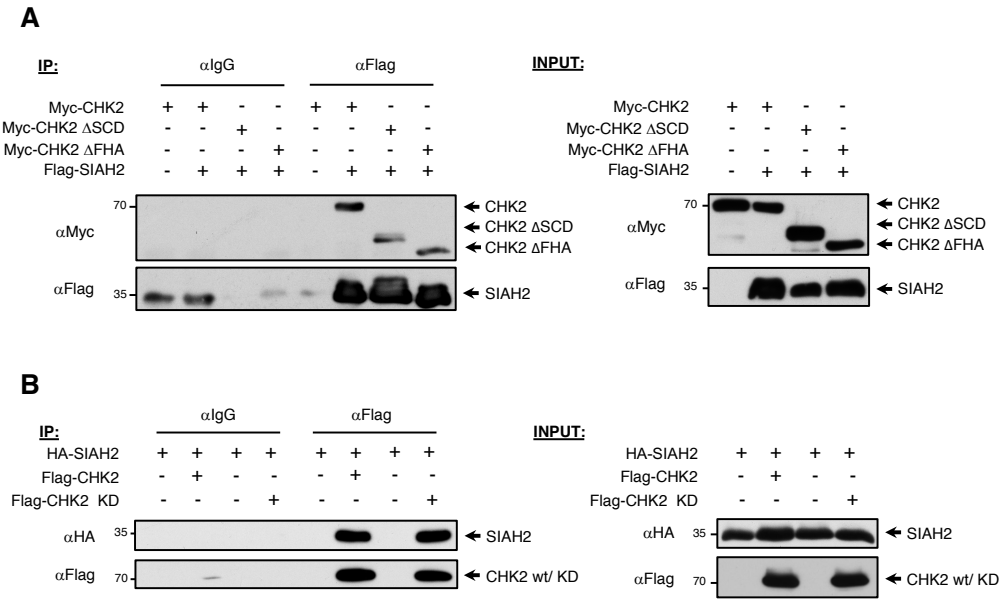

Figure 13. Comparation of the interaction of SIAH2 with different CHK2 forms. (A) HEK-293T cells were transfected with expression plasmids encoding Flag-tagged SIAH2, Myc-CHK2 and FHA or SCD domain-deleted mutants as indicated, and after 36 h the proteasome inhibitor MG-132 (10 μM) was added for another 12 h to avoid SIAH2 autodegradation. Cells were lysed, subjected to immunoprecipitation (IP), and after elution detected by WB. A small fraction (5%) of the lysate was tested for the occurrence of the indicated proteins by immunoblot (INPUT). **(B)** HEK-293T cells were transfected with expression plasmids indicated, stimulated with MG-132 during 12 h, lysed and subjected to IP using anti-Flag antibody. Protein expression was detected by western blotting with the indicated antibodies. A fraction of the lysate was used to test the expression of the indicated proteins (INPUT). We show a representative blot of three independent experiments.

6.1.3. SIAH2 mediates CHK2 ubiquitination and proteosomal degradation

Based on the ability of SIAH2 to lead the ubiquitination and proteosomal degradation of many of its interaction partners, we analyzed whether SIAH2 can degrade CHK2 through an ubiquitin/proteasome-dependent process. We coexpressed CHK2 with increasing amounts of SIAH2 or the ligase-deficient SIAH2 point mutant (SIAH2 RM). Expression of SIAH2 resulted in a dose-dependent decrease in CHK2 protein levels in HEK-293T cells (Figure 14A). Similar results were obtained in MOR and A549 cells (Figure 14B). By contrast, CHK2 levels were not altered in the presence of the SIAH2 RM. A comparative assay with SIAH1 showed that SIAH2 is the only

member of the human family able to induce CHK2 degradation (Figure 14C). Then, we examined this effect in the presence or absence of the proteasome inhibitor MG-132. The addition of this inhibitor stabilized SIAH2 and significantly prevented CHK2 degradation (Figure 14D).

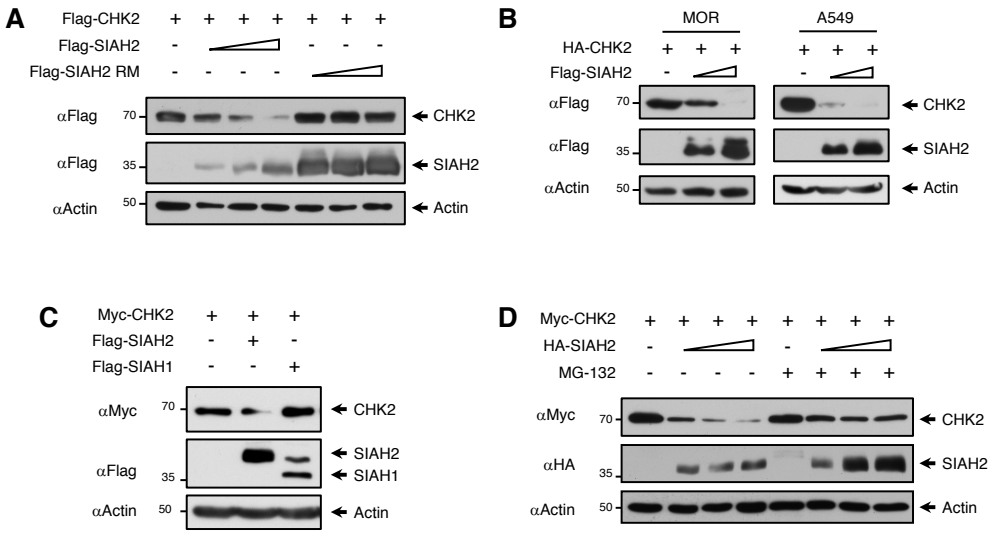

Figure 14. SIAH2 mediates proteasomal degradation of CHK2. (A) HEK-293T cells were transfected to express CHK2 and the levels were evaluated in response to increasing concentrations of SIAH2 wild type or SIAH2 RING mutant by WB. We show a representative blot of three independent experiments. **(B)** MOR and A549 cells were transfected to express CHK2 and its levels were evaluated in response to increasing concentrations of SIAH2 wild type by WB. **(C)** HEK-293T cells were transfected to express CHK2 in the presence of SIAH1 or SIAH2. After 36 h cells were lysed and protein expression analyzed by immunoblot. **(D)** Cells were transfected with the indicated plasmids to express CHK2 and increasing concentrations of SIAH2 in the presence or absence of MG-132. Cells were lysed and protein expression analyzed by WB. We show a representative blot of three independent experiments.

Next, we decided to analyze the impact of SIAH2 expression at different levels on the endogenous CHK2. Increasing amounts of SIAH2 revealed a dose-dependent decrease in CHK2 protein levels without affect mRNA expression (Figure 15A). Then we analyzed the effect of SIAH2 inhibition by specific siRNA together with the effect of ETP stimulation, based on previous results by which DNA damage partially disrupted SIAH2-CHK2 interaction. SIAH2 depletion increases CHK2 levels (Figure 15B) as well as its half-life (Figure 15C), indicating that CHK2 basal levels can be regulated by this E3 ligase. In addition, ETP stimulation increased CHK2 stability as well as its half-life

(Figure 15C), which were not affected by SIAH2 inhibition. These changes were accompanied by an increase in CDC25C-Ser216 phosphorylation. Taken together, these results suggest that SIAH2 regulates CHK2 turnover through a mechanism occurring at the protein level, affecting its stability and activity.

We next examined the effect of SIAH2 on CHK2 ubiquitination *in vivo* and *in vitro*. We coexpressed His-Ubiquitin and HA-CHK2 with or without different concentrations of SIAH2 in the presence of MG-132, and analyzed the ubiquitination status of CHK2. As shown in Figure 16A, CHK2 polyubiquitination became more evident in the presence of increasing concentrations of SIAH2. By contrast, CHK2 polyubiquitination was inhibited in the presence of the SIAH2 RM (Figure 16B). Then, we examined the effect of SIAH2 deficiency on the basal level of CHK2 polyubiquitination, comparing control MEFs to Siah1a$^{-/-}$/Siah2$^{-/-}$ MEF cells. Ubiquitination levels of CHK2 were significantly lower in the knockout MEFs lacking Siah1a and Siah2 (Figure 16C). Finally, we analyzed SIAH2 ability to directly ubiquitylate CHK2 through an *in vitro* ubiquitination assay. Polyubiquitination of CHK2 was observed only in the presence of SIAH2 (Figure 16D).

CHK2 phosphorylation is a relevant mechanism for the control of its activity and stability. After DNA damage, T68 phosphorylation leads to conformational changes that induce dimerization and full activation (Ahn *et al.*, 2002; Xu *et al.*, 2002). Similarly, S456 phosphorylation is critical for the stability control by ubiquitination (Bohgaki *et al.*, 2013; Kass *et al.*, 2007; Kass *et al.*, 2009). We decided to evaluate the effect of SIAH2 on CHK2 wt compared to CHK2 S456A and T68A mutants. As shown in Figures 17A and 17B, both mutants were degraded in a similar way to CHK2 wt, indicating that the phosphorylation status of these residues is not relevant for the degradation mediated by SIAH2. Moreover, we compared the interaction between SIAH2 and CHK2 wt in the presence or absence of ATM-ATR inhibitor caffeine, or CHK2 S19A and T68A mutants by coimmunoprecipitation assays. These experiments revealed that CHK2 phosphorylation status, at least in these important residues, is not relevant for the association with SIAH2 (Figure 17C). In the same sense, we evaluated the capacity of SIAH2 to degrade FHA or SCD domain-deleted mutants (Figure 17D). Although FHA

domain-deleted mutant was partially degraded, the expression of the SCD domain-deleted mutant was not affected by SIAH2.

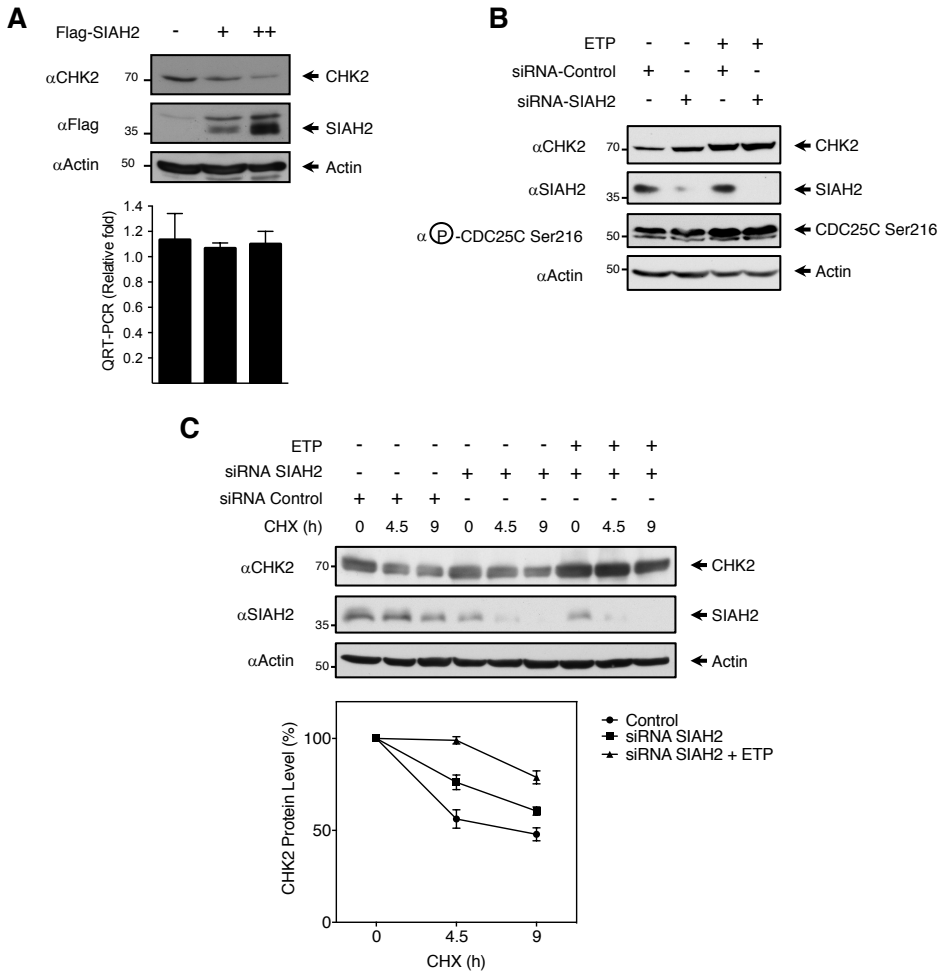

Figure 15. SIAH2 degrades CHK2 at endogenous levels. (A) HEK-293T cells were transfected to express increasing amounts of SIAH2, harvested and lysed. One fraction was used to analyze endogenous CHK2 protein levels while another aliquot was used to analyze CHK2 mRNA levels by qPCR. Data are mean ± SD of n = 3. We show a representative blot of three independent experiments. Primer sequences are available upon request. **(B)** HEK-293T cells were transfected with SIAH2 or scrambled (control) siRNAs, after 4 days stimulated with etoposide (10 µM) during 24 h as indicated, lysed and protein expression analyzed by immunoblot. We show a representative blot of three independent experiments. **(C)** HEK-293T cells were transfected with SIAH2 or scrambled (control) siRNAs and, after 4 days of culture, treated with the protein synthesis inhibitor cycloheximide (CHX) (40 µg/ml) for 4.5 and 9 h in the presence or absence of ETP (10 µM). Cell lysates were analyzed by immunoblot using actin expression as the loading control. The graph represents the mean ± SD of band density from 3 different experiments.

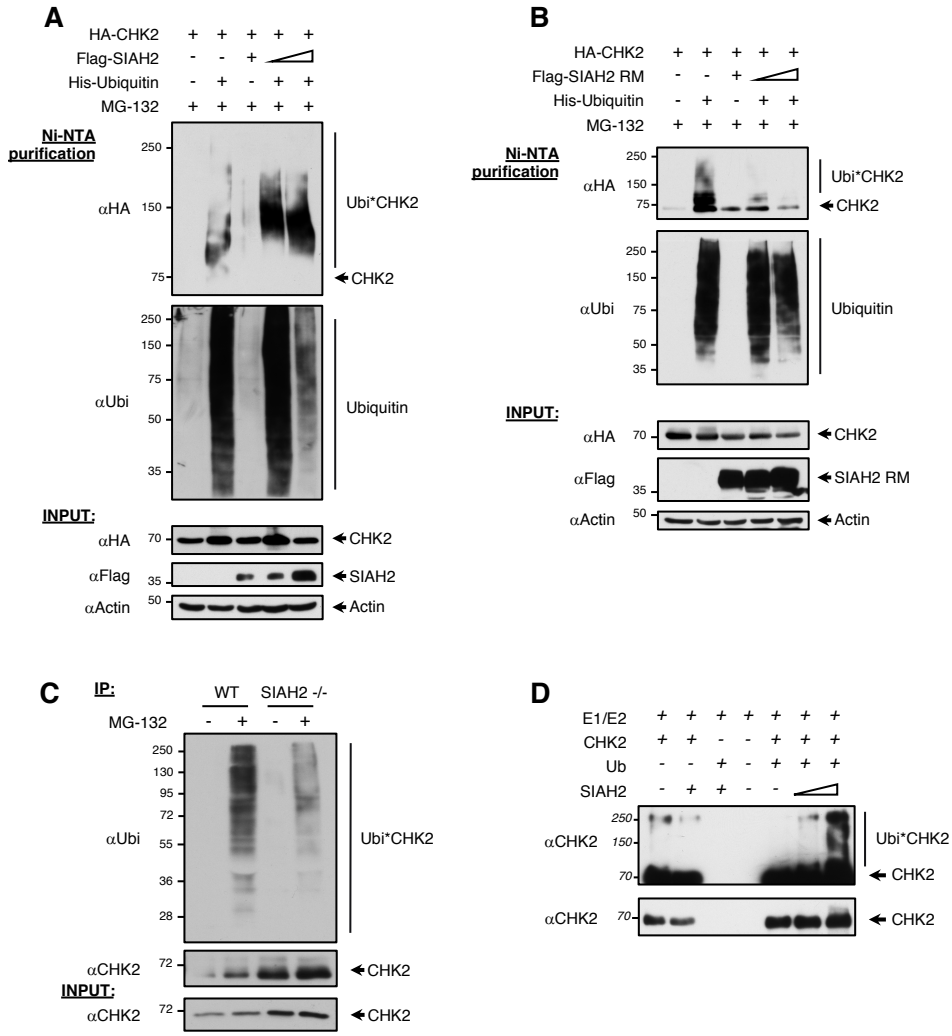

Figure 16. CHK2 ubiquitination *in vivo* and *in vitro* by SIAH2. (A) HEK-293T cells were transfected with expression plasmids encoding HA-CHK2, Flag-SIAH2 and His-Ubiquitin. After 36 h cells were incubated in the presence of MG-132 (10 μM) during 12 h and lysed under denaturing conditions. His-tagged ubiquitin was purified with Ni-NTA agarose columns and ubiquitinated CHK2 was analyzed by WB. A fraction was tested for the occurrence of the indicated proteins (INPUT). We show a representative blot of three independent experiments. **(B)** The experimental settings were similar to (A) with the exception that Flag-SIAH2 RING mutant was transfected instead of the wild type form of the ligase. **(C)** Wild type and Siah1a$^{-/-}$/Siah2$^{-/-}$ MEF cells were stimulated or not with MG-132 during 12h, lysed and subjected to immunoprecipitation using anti-CHK2 antibody. The precipitates were subjected to WB analysis with anti-CHK2 or anti-Ub antibodies. A small fraction of the lysate was tested for the occurrence of CHK2 (INPUT). We show a representative blot of three independent experiments. **(D)** *In vitro* ubiquitination of CHK2 with E1, E2 (UbcH5c), Ubiquitin and SIAH2 recombinant human proteins. Upon activation with ATP, the samples were incubated for 2 h at 30 ºC, and the reaction stopped by the addition of SDS-loading buffer. Ubiquitinated CHK2 proteins were visualized by WB using anti-CHK2 antibody.

Finally, we decided to study the effect of DNA damage on CHK2 degradation by SIAH2. ETP stimulation prevented CHK2 polyubiquitination (Figure 18A) and degradation by SIAH2 (Figure 18B), as well as increased its half-life (Figure 15C). Similar results were obtained after stimulation with cisplatin and 5-FU (Figure 18C). Collectively, these data show that SIAH2 stimulates basal CHK2 polyubiquitination, thereby identifying CHK2 as a substrate for SIAH2-mediated ubiquitination and proteasomal degradation.

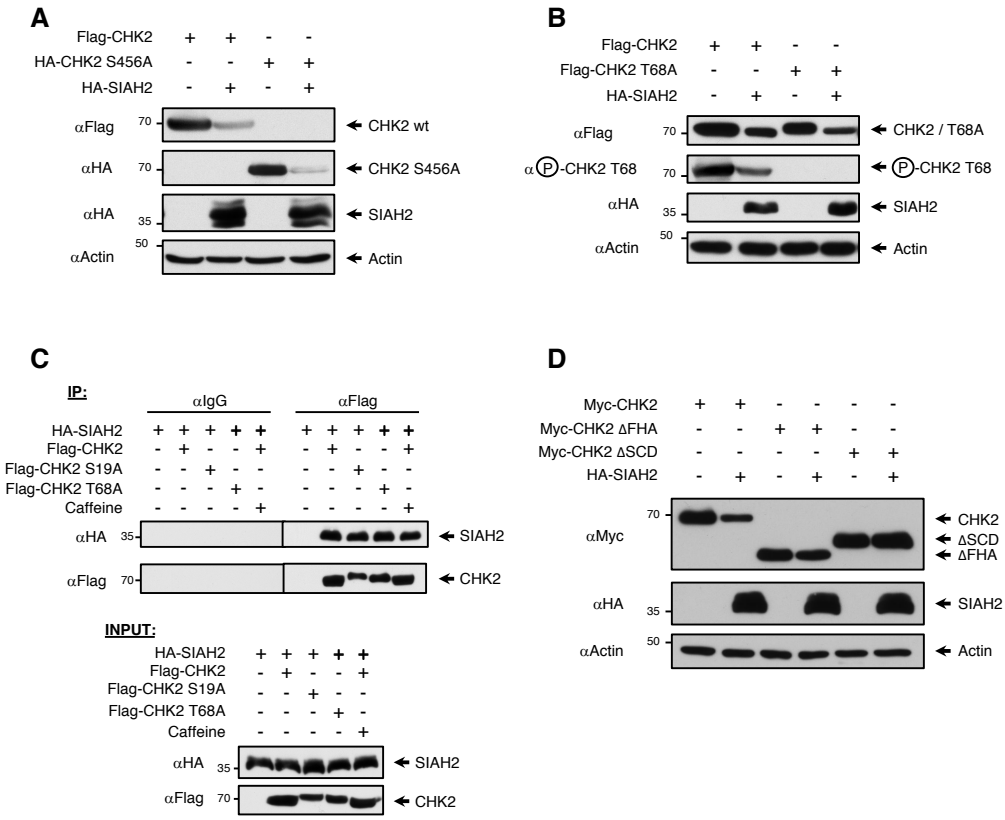

Figure 17. Effect of SIAH2 on CHK2 under its activation and stabilization. HEK-293T cells were transfected to express CHK2 wild type and the S456A **(A)** or the T68A mutant **(B)** in the presence or absence of SIAH2. After 36 h cells were lysed and protein expression analyzed by immunoblot with the indicated antibodies. **(C)** HEK-293T cells were transfected with the indicated plasmids, after 36 h stimulated with MG-132 for another 12 h and caffeine (3 mM) for another 2 h as indicated. Cells were lysed, subjected to immunoprecipitation (IP) and, after elution, protein expression was analyzed by immunoblot. A small fraction (5%) of the lysate was tested for the occurrence of the indicated proteins (INPUT). **(D)** HEK-293T cells were transfected with expression plasmids encoding HA-SIAH2, Myc-CHK2 and FHA or SCD domain-deleted mutants as indicated, after 36h cells were lysed and subjected to WB with the indicated antibodies.

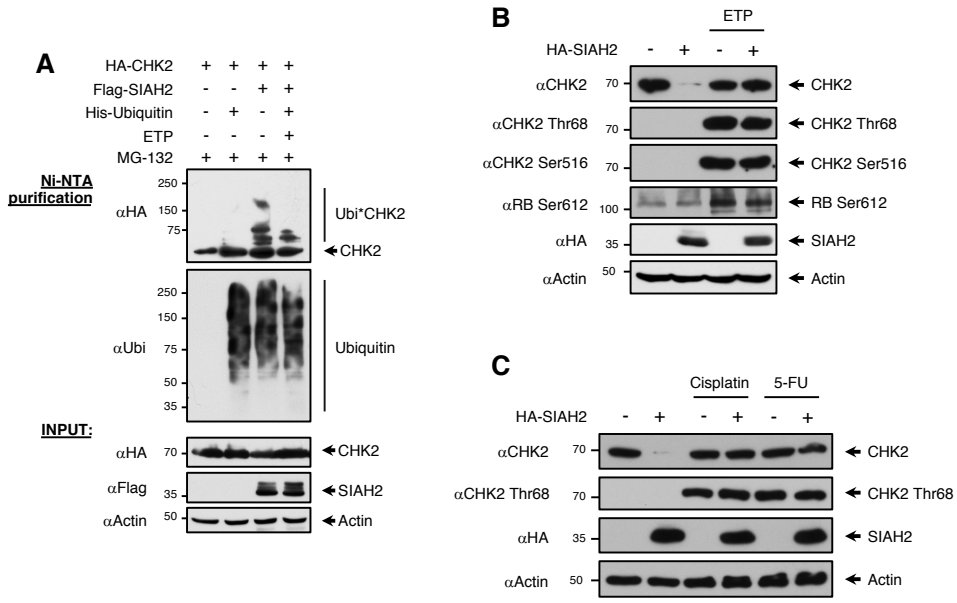

Figure 18. Effect of DNA damage on CHK2 degradation by SIAH2. (A) HEK-293T cells were transfected with the indicated plasmids and, 24 h post-transfection, stimulated with ETP (10 μM) during 24 h and with MG-132 10 μM during 12 h. Cells were lysed under denaturing conditions, His-tagged ubiquitin was purified with Ni-NTA agarose columns and ubiquitinated CHK2 was analyzed by WB. An aliquot of the input material was also tested for correct protein expression (INPUT). **(B)** HEK-293T cells were transfected or not to express SIAH2, and 24 h post-transfection stimulated with ETP (10 μM) during 24 h as indicated. Cells were lysed and protein expression was analyzed by immunoblot with the indicated antibodies. We show a representative blot of three independent experiments **(C)** The experimental settings were similar to (B) with the exception that cells were stimulated with cisplatin (5 μM) or (5-FU, 0.5 μg/ml) as indicated.

6.1.4. CHK2 phosphorylates SIAH2 in at least three residues *in vivo* and *in vitro*

As we showed in previous works (Calzado *et al.*, 2009; Perez *et al.*, 2012), some kinase substrates of SIAH2 have the ability to phosphorylate this E3 ligase. Therefore, we tested the capacity of CHK2 to phosphorylate SIAH2. CHK2 overexpression resulted in the presence of slower migrating SIAH2 bands, an effect not observed with CHK2 kinase dead mutant (KD) (Figure 19A). SIAH2 phosphorylation was analyzed with a specific phospho-SIAH2 antibody for Ser28 (Calzado *et al.*, 2009). To confirm that the upshifted SIAH2 bands were phosphorylated forms, we incubated cell extracts with λ phosphatase. Treatment transformed the slower electrophoretic

mobility of SIAH2 bands into the faster migrating bands, and SIAH2 phosphorylation disappeared (Figure 19B). These results indicate that the upshifted SIAH2 bands observed after CHK2 wild-type overexpression represent hyperphosphorylated forms.

To examine the capacity of CHK2 to directly phosphorylate SIAH2, we performed an *in vitro* kinase assay. The presence of GST-CHK2 showed the occurrence of an upper band of GST-SIAH2 detected with a specific phospho-SIAH2 antibody (Figure 19C), which disappeared after λ phosphatase treatment (Figure 19D). Next, we evaluated the CHK2 ability to phosphorylate endogenous SIAH2 by comparing phospho-SIAH2 endogenous levels of control cells to the cells where endogenous CHK2 was knocked down by siRNA treatment (Figure 20A). CHK2 inhibition caused a significant reduction of endogenous SIAH2 Ser28 phosphorylation. Taken together, these results clearly indicate that CHK2 can directly phosphorylate SIAH2. Once again, we examined the effect of ETP stimulation on SIAH2 phosphorylation by CHK2. As shown in Figure 20B and coherent with the previous results, stimulation with ETP decreased substantially SIAH2 phosphorylation levels mediated by CHK2.

To identify the SIAH2 sites phosphorylated by CHK2, we used four different phospho-specific antibodies (Thr26, Ser28, Ser68 and Thr119) previously described (Perez *et al*., 2012). SIAH2 or point mutants of the phosphorylated amino acids to alanine were expressed either alone or together with CHK2, followed by detection of single-site phosphorylation (Figure 20C). CHK2 expression caused phosphorylation in 3 different sites (Thr26, Ser28 and Ser68), but we did not observe any significant changes in Thr119. Ser28 mutation precluded phosphorylation at Thr26, probably due to the fact that these sites are very close and in the same epitope. Overall, these results clearly indicate that CHK2 directly phosphorylates SIAH2 at a minimum of three residues.

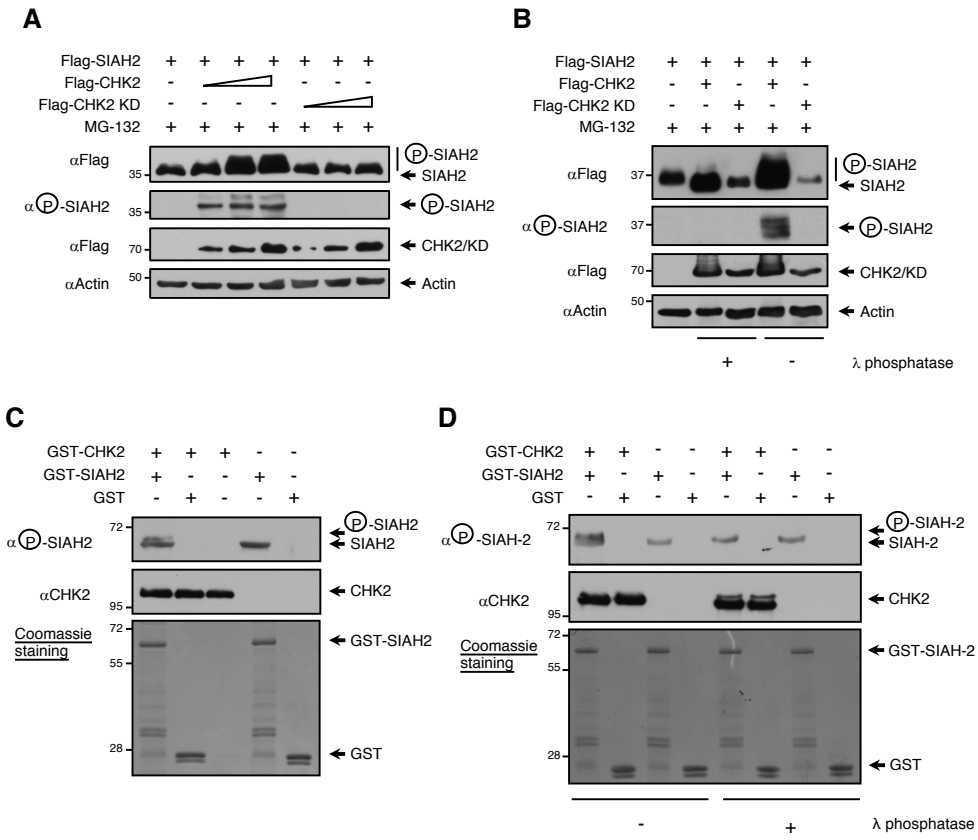

Figure 19. CHK2 phosphorylates SIAH2 *in vivo* and *in vitro*. (A) HEK-293T cells were transfected to express Flag-SIAH2 and increasing amounts of Flag-CHK2 wild type or Flag-CHK2 kinase dead mutant (KD), and after 36 h, they were treated for 12 h with the proteasome inhibitor MG-132 (10 μM). Cell lysates were analyzed by immunoblotting with the indicated antibodies. SIAH2 phosphorylation was seen by the upshifted band and also by the use of a specific antibody recognizing phosphorylated Ser28 of SIAH2. We show a representative blot of three independent experiments. **(B)** HEK-293T cells were transfected with the indicated plasmids and treated with MG-132 for 12 h. Cells were lysed in phosphatase inhibitor-free buffer in the absence or presence of λ-phosphatase. Electrophoretic mobility was determined by immunoblotting and in parallel SIAH2 phosphorylation was analyzed with phospho-specific antibody for Ser28 of SIAH2. We show a representative blot of three independent experiments. **(C)** *In vitro* kinase assay was performed with active CHK2 recombinant protein and purified GST-SIAH2. Phosphorylation was determined with phospho-specific antibody for Ser28 of SIAH2, while protein levels were visualized by Coomassie staining (lower). **(D)** *In vitro* kinase assay was performed with active CHK2 recombinant protein and purified GST-SIAH2. The products of the reaction were treated or not with λ-phosphatase, and phosphorylation was determined with a phospho-specific antibody for Ser28 of SIAH2. Coomassie staining of the GST proteins is shown in lower panel.

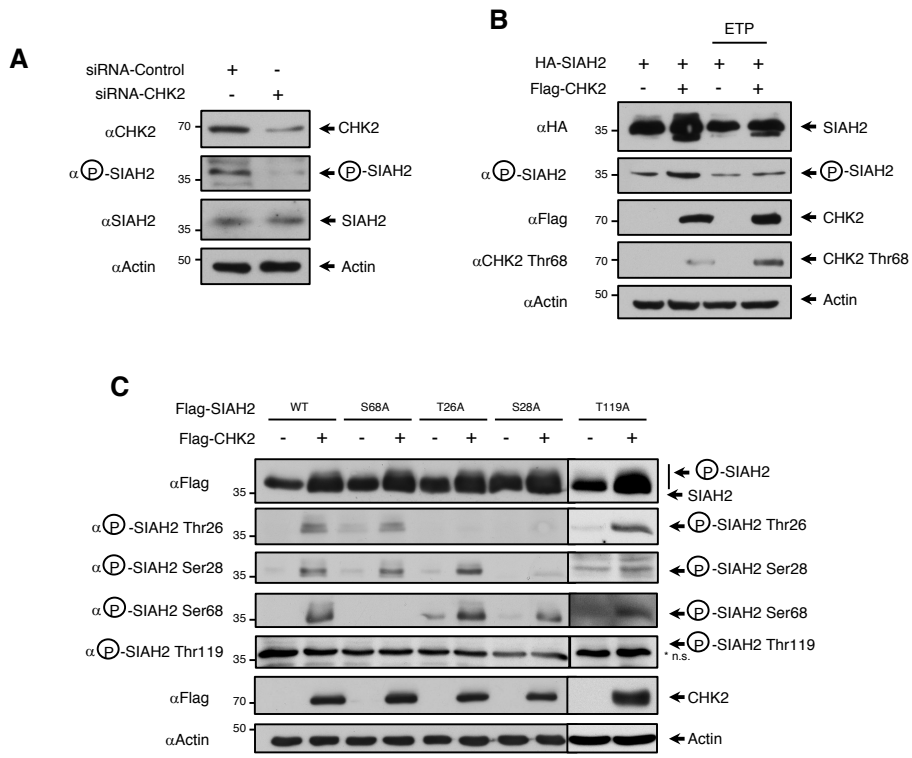

Figure 20. CHK2 phosphorylates SIAH2 in at least three residues. (A) HEK-293T cells were transfected with CHK2 or scrambled (control) siRNAs, lysed after 4 days of culture and CHK2 and SIAH2 phosphorylation analyzed by WB. We show a representative blot of three independent experiments. **(B)** Cells were transfected to express HA-SIAH2 in the presence or absence of Flag-CHK2. 24h post-transfection cells were stimulated with ETP (6 μM) during 24 h, lysed and protein expression was analyzed by immunoblot with the indicated antibodies. SIAH2 phosphorylation was analyzed with phospho-specific antibody for Ser28. We show a representative blot of three independent experiments. **(C)** HEK-293T cells were cotransfected with Flag-SIAH2 or the indicated SIAH2 point mutants either alone or along with CHK2 and treated with MG-132 for 12 h. Electrophoretic mobility was determined by WB and in parallel SIAH2 phosphorylation was analyzed with phospho-specific antibodies for the residues indicated. We show a representative blot of three independent experiments.

6.1.5. Effect of SIAH2 phosphorylation on CHK2 turnover via ubiquitination and proteosomal degradation

Does SIAH2 phosphorylation by CHK2 control the degradation of this kinase through the ubiquitin/proteasome system? To address this issue, we transfected HEK-293T cells with Flag-CHK2 or Flag-CHK2 KD with increasing amounts of Flag-SIAH2. CHK2 degradation by SIAH2 was independent of the presence of its kinase activity (Figure 21A). Next, we compared the capacity of the phosphorylation (SIAH2-3A) and

the phosphomimic (SIAH2-3D) mutants in the residues phosphorylated by CHK2 to degrade this kinase. Cells were transfected with Flag-CHK2 together with Flag-SIAH2-WT, Flag-SIAH2-3A or Flag-SIAH2-3D, using Flag-SIAH2 RM as a negative control. As shown in Figure 21B, CHK2 was degraded to a similar way in the three cases. These results support the hypothesis that the phosphorylation state of SIAH2 does not alter its capacity to degrade CHK2.

Others and we have revealed how SIAH2 phosphorylation affects its activity on certain substrates. Although the former results indicated that this is not the case for CHK2, we decided to analyze the effect on other substrates as prolyl hydroxylase 3 (PHD3). SIAH2 regulates Hypoxia-inducible factor 1-alpha (HIF-1α) expression mostly via PHD3 inhibition (Nakayama *et al.*, 2004). Cells were transfected with Flag-PHD3, together with different SIAH2 variants in the presence or absence of Flag-CHK2, and a luciferase construct controlled by the HIF-1-dependent erythropoietin promoter (Epo-Luc). SIAH2 produced an important reduction in PHD3 expression, which was reflected in a significant induction of HIF-dependent Epo-Luc promoter activity. SIAH2-3A mutant showed a reduced capacity to degrade PHD3, reflected in a lower response to the Epo-Luc promoter. On the contrary, the phosphomimetic SIAH2-3D mutant showed a strongly increased ability to degrade PHD3 and increase the Epo-Luc promoter response (Figure 21C). Coexpression of CHK2 strongly diminished PHD3 inhibition by SIAH2 and showed a significant reduction in the Epo-Luc promoter response. These experiments demonstrate that the SIAH2 phosphorylation mediated by CHK2 can regulate its capacity to degrade PHD3.

We subsequently investigated the effects of SIAH2 phosphorylation state in response to CHK2 on its capacity to degrade other two relevant SIAH2 substrates as DYRK2 and HIPK2. HEK-293T cells were transfected with kinase inactive mutants Flag-DYRK2 KD (Figure 22A) or Flag-HIPK2 KD (Figure 22B) in order not to interfere with SIAH2 phosphorylation state, together with SIAH2 in the presence or absence of full-length CHK2, FHA or SCD domain-deleted mutants. In agreement with the PHD3 results, the presence of CHK2 significantly reduced HIPK2 and DYRK2 degradation. By contrast, FHA or SCD domain-deleted mutants, which lack the ability to phosphorylate

SIAH2 (Figure 22C), did not change the levels of both kinases. Altogether these data suggest that SIAH2 phosphorylation mediated by CHK2 does not affect its capacity to degrade this kinase, but modulates its ability to regulate other substrates such as PHD3, DYRK2 and HIPK2.

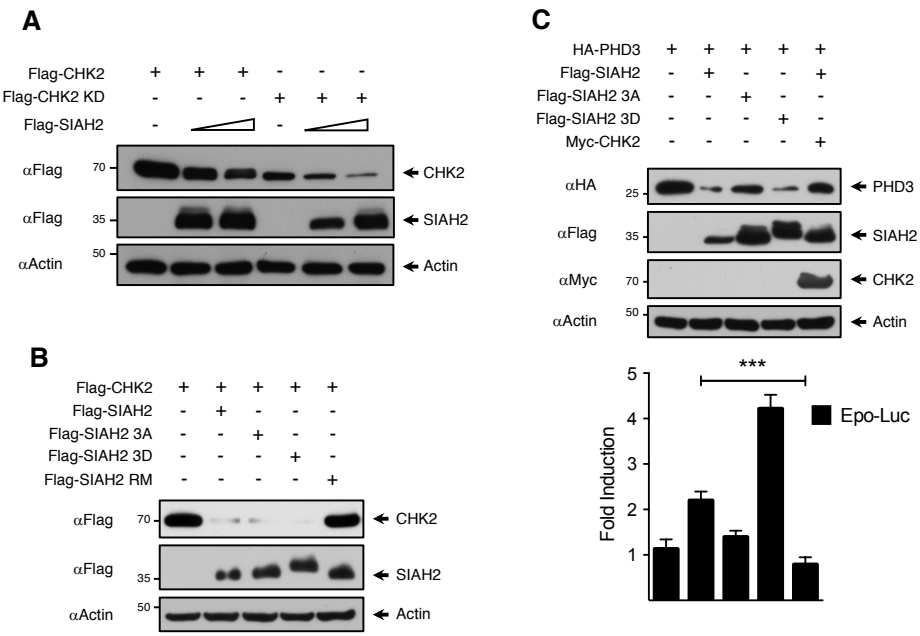

Figure 21. The phosphorylation state of SIAH2 does not alter its capacity to degrade CHK2. (A) HEK-293T cells were transfected to express Flag-CHK2 or Flag-CHK2 KD (Kinase mutant) with increasing amounts of SIAH2, lysed and protein expression was analyzed by immunoblot with the indicated antibodies. We show a representative blot of four independent experiments. **(B)** Cells were transfected with Flag-CHK2 and the plasmids encoding SIAH2 wild type and the phosphorylation (SIAH2-3A) and the phosphomimic (SIAH2-3D) mutants in the residues Thr26, Ser28 and Ser68. After 36 h cells were lysed and the stability of CHK2 was revealed by immunoblotting. We show a representative blot of three independent experiments. **(C)** HEK-293T cells were transfected with the indicated plasmids together with a luciferase construct controlled by the HIF-1-dependent erythropoietin promoter (Epo-Luc). After 36 h, cells were lysed and further analyzed for the indicated proteins by immunoblots (upper panel) or for luciferase expression (lower panel). Data are mean ± SD of n = 3 experiments. *** $P < 0.001$. We show a representative blot of three independent experiments.

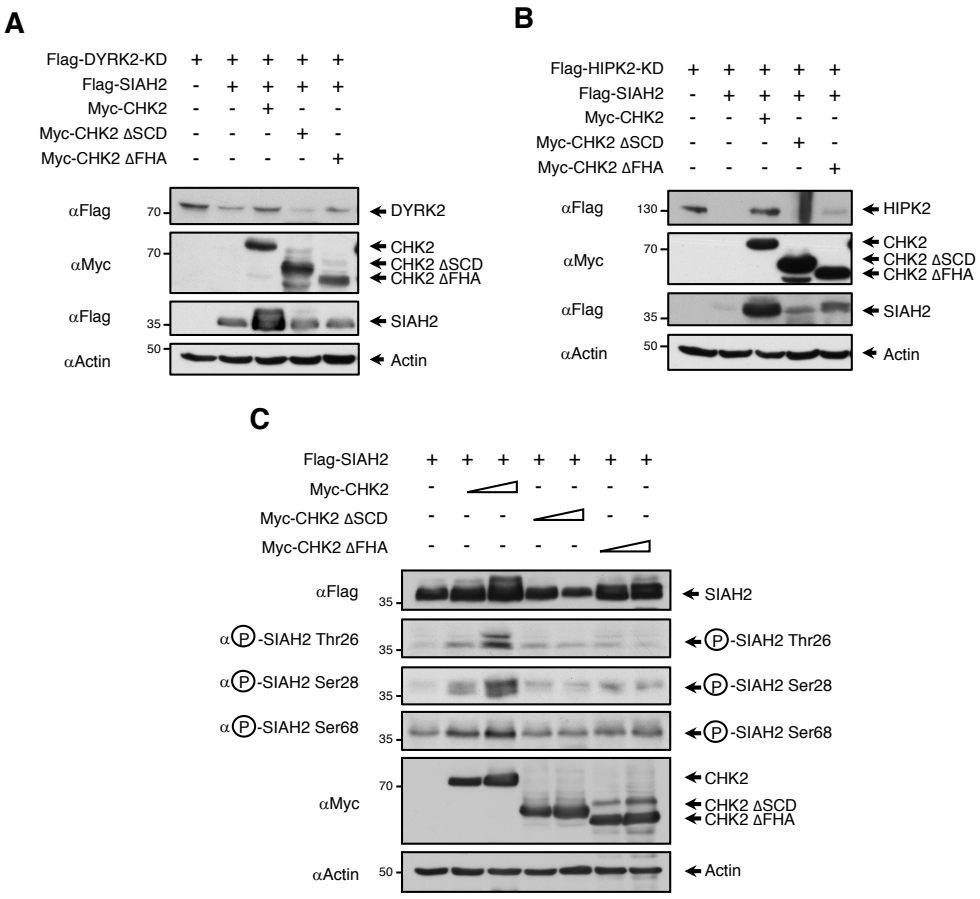

Figure 22. Regulation of SIAH2 activity by CHK2. **(A)** and **(B)** HEK-293T cells were transfected with the indicated plasmids, and after 36 h lysed and protein expression evaluated by immunoblot with the indicated antibodies. We show a representative blot of three independent experiments. **(C)** HEK-293T cells were transfected to express SIAH2 together with different amounts of CHK2 wild type or FHA and SCD domain-deleted mutants as indicated. Electrophoretic mobility was determined by WB and in parallel SIAH2 phosphorylation was analyzed with phospho-specific antibodies for the residues indicated.

6.1.6. Effect of SIAH2 on CHK2 capacity to regulate cell cycle arrest or apoptosis in response to DNA damage

CHK2 kinase has a relevant role in the DNA damage response through cell-cycle checkpoint regulation (Zannini *et al.*, 2014). Therefore, we tested the ability of SIAH2 to affect cell-cycle checkpoint activation in response to DNA damage. HEK-293T

cells were transfected or not with SIAH2 and treated or not with ETP during 12 hours. This time was taken to observe the direct effect on the cell cycle arrest, which was analyzed together with the expression of CHK2, CDC25C and SIAH2 proteins. As depicted in Figure 23, SIAH2 expression resulted in a clear reversion of the cellular arrest in G2/M phase induced by the stimulation with ETP, presenting an inhibition of total and phosphorylated CHK2 (Thr68 and Ser516) and a reduction in phospho-CDC25C-Ser216 protein (CHK2 substrate).

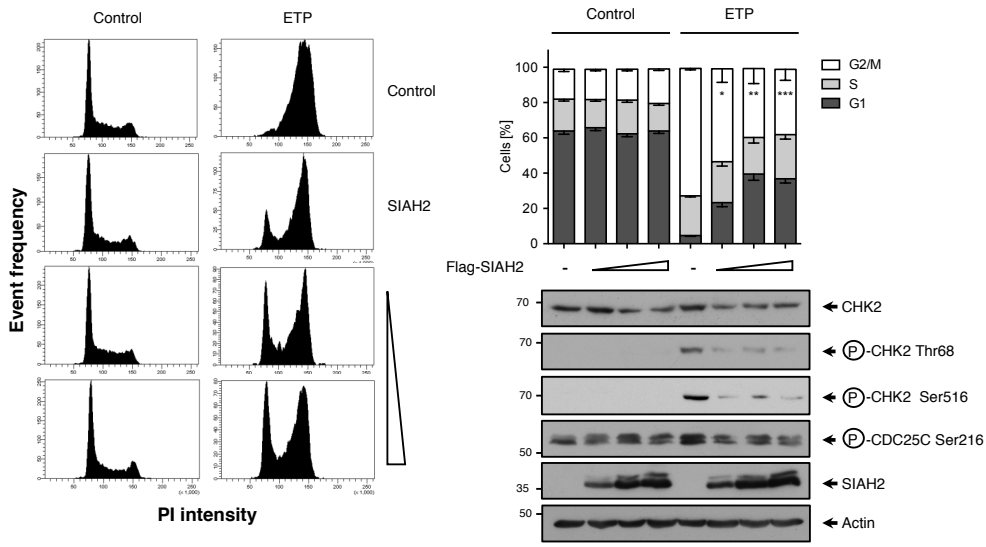

Figure 23. SIAH2 reverts cell cycle arrest caused by DNA damage. HEK-293T cells were transfected with increasing amounts of SIAH2 and after 12 h stimulated with ETP (6 μM) during 12 h. A fraction of the cells was used for cell cycle analysis (left panel) using flow cytometry. Left panel shows representative cell cycle distributions. Right panel shows quantitation of percentages of the cells in each phase of the cell cycle. Data are mean ± SD of n = 3 experiments. Another aliquot (lower panel) was lysed and protein expression was analyzed by immunoblot with the indicated antibodies. We show a representative blot of three independent experiments.

We then investigated whether SIAH2 contributes to the apoptosis induced by DNA damage and the CHK2 role on this effect. HeLa cells were transfected with the indicated plasmids and stimulated with ETP. As shown in Figure 24A, apoptosis percentage induced by stimulation with ETP was reduced in response to SIAH2 expression. In parallel, a significant reduction in the CHK2 expression was observed. Restoration of CHK2 protein expression to its endogenous levels was associated with a

significant recovery of apoptosis. Cell cycle phase analysis was performed in parallel (Figure 24B).

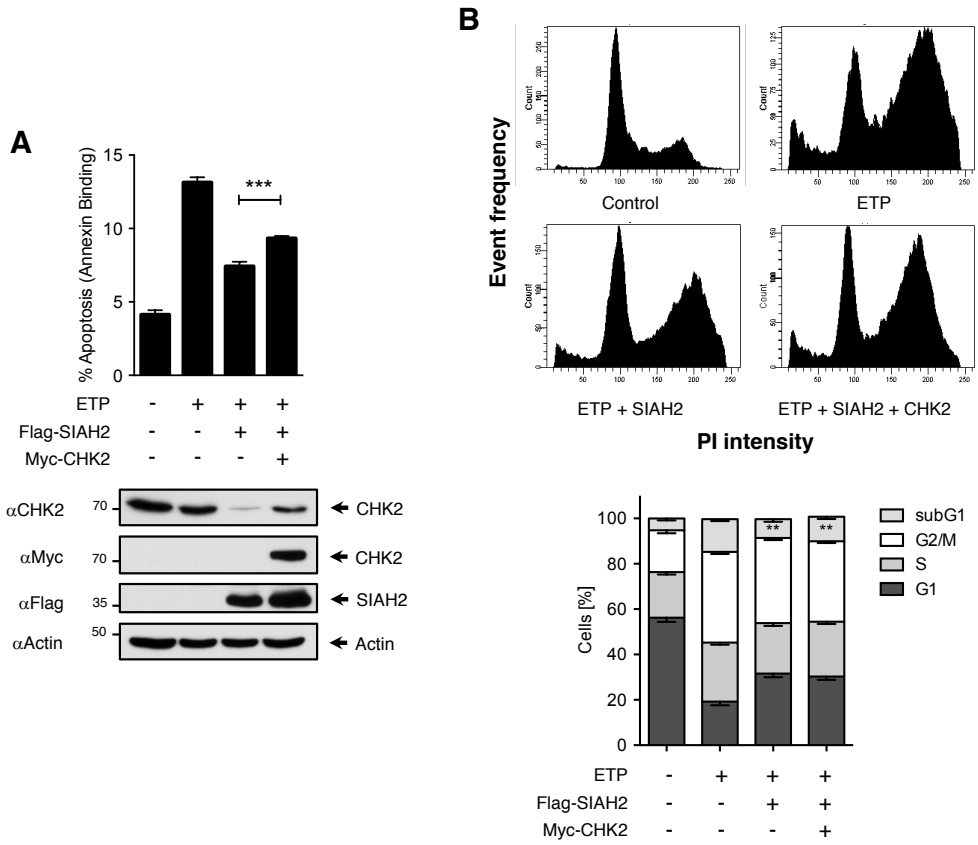

Figure 24. Role of SIAH2-CHK2 complex in the apoptosis induced by DNA damage. (A) HeLa cells were transfected with the indicated plasmids and 24 h later stimulated with ETP (10 μM) for another 24 h. One aliquot was used for Apoptosis analysis determined by flow cytometry using Annexin V/PI Staining (Upper panel), while another fraction was lysed and the levels of the indicated protein analyzed by immunoblot. Data are mean ± SD of n = 3 experiments. *** $P < 0.001$. **(B)** HeLa cells were transfected with the indicated plasmids and 24 h later stimulated with ETP 10 μM for another 24 h. Cell cycle was analyzed by flow cytometry. Upper panel shows representative cell cycle distributions. Lower panel shows quantitation of percentages of the cells in each phase of the cell cycle. Data are mean ± SD of n = 3 experiments.

In addition, we observed that SIAH2 expression increases cell viability in response to cytotoxic drugs (5-FU or Oxaliplatin) in HEK-293T (Figure 25A) and HeLa cells (Figure 25B). Overall, these results indicate the SIAH2 ability to partially regulate the CHK2 capacity to control cell cycle arrest or apoptosis in response to DNA damage.

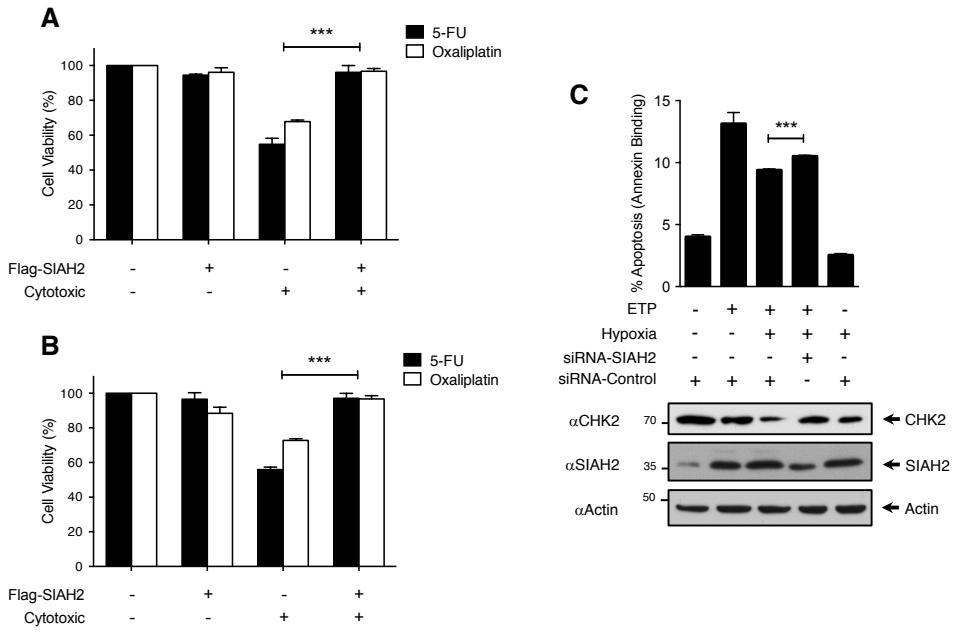

Figure 25. Functional consequences of CHK2 regulation by SIAH2. HEK-293T cells **(A)** and HeLa cells **(B)** were transfected or not to express SIAH2 and 24 h later stimulated as indicated with 5-fluorouracil (5-FU, 0.5 μg/ml) or Oxaliplatin (0.5 μM) during 24 h. Cell viability was measured by MTT assay. Data are mean ± SD of n = 3 experiments. * $P = 0.01$; ** $P = 0.002$; *** $P < 0.001$. **(C)** HeLa cells were transfected with the indicated siRNA and 24 h later stimulated with ETP 10 μM for another 24 h. Cells were subjected to hypoxia (1% O_2) during 12 h and one fraction was used for apoptosis analysis by Annexin V/PI Staining (Upper panel), while another fraction was lysed and the levels of the indicated protein analyzed by immunoblot. Data are mean ± SD of n = 3 experiments. *** $P < 0.001$.

Finally, we decided to study the functional consequences of CHK2-SIAH2 modulation in the context of hypoxia. SIAH2 is an essential component of the hypoxia response pathway with relevant implications on the carcinogenesis control (Nakayama *et al.*, 2004; Wong and Moller, 2013). Moreover, one of the causes of hypoxic tumor resistance to treatment is the hypoxia ability to alter the DNA damage response-signaling pathway (Bristow and Hill, 2008). To test this hypothesis, we performed apoptosis analyses in HeLa cells that were subjected to hypoxia, stimulated with ETP and transfected or not with specific siRNA for silencing endogenous SIAH2. Hypoxia reduced the percentage of apoptosis in response to ETP, increasing SIAH2 levels and significantly reducing CHK2 expression. Moreover, SIAH2 depletion increased CHK2 levels, which was accompanied by a significant recovery of apoptosis (Figure 25C).

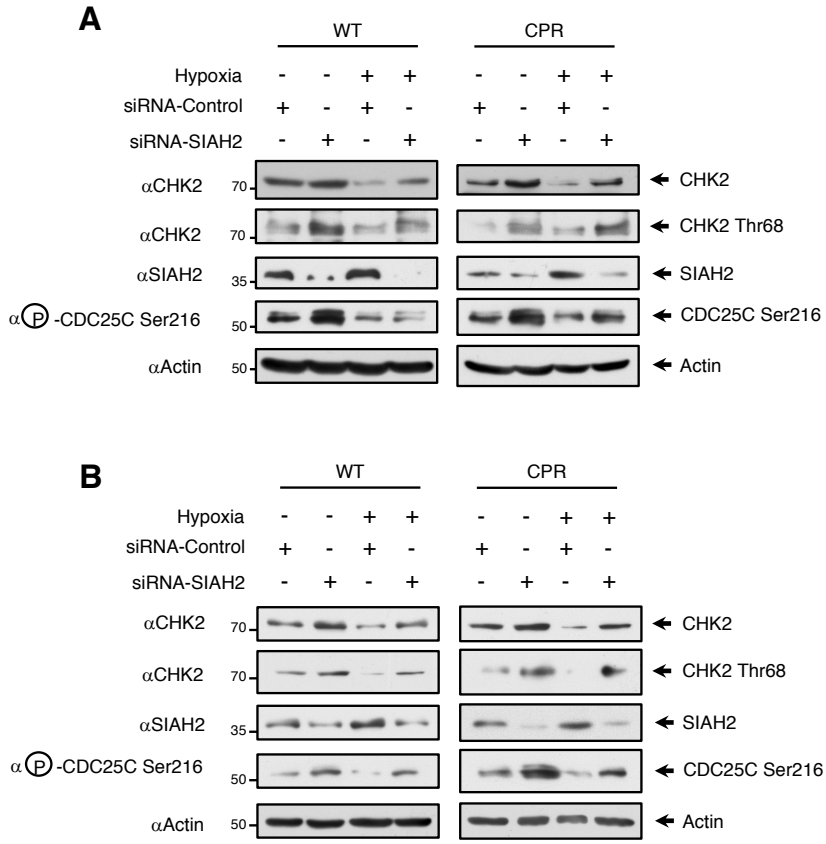

Figure 26. Functional consequences of CHK2 regulation by SIAH2 in lung cancer cell lines. (A) MOR wt and cisplatin-resistant (CPR) cells or **(B)** A549 wt and cisplatin-resistant (CPR) cells were transfected with SIAH2 or scrambled (control) siRNAs in the absence of cisplatin and after 4 days were subjected to hypoxia (1% O_2) during 12 h as indicated. Cells were lysed and protein expression was evaluated by immunoblot with the indicated antibodies.

Similar experiments were performed in A549 and MOR cells where, again, hypoxia produced a clear decrease in total and Thr68 CHK2 levels, thereby affecting its activity on CDC25C. SIAH2 knockdown under hypoxia conditions produced a significant increase in CHK2, which was also reflected in its activity (Figures 26A and 26B). Similar results were obtained in the same cisplatin-resistant cell lines, thus indicating that this regulation is conserved in at least these two cell types. Collectively, all these results conclusively show that hypoxia regulates, at least partially, CHK2 stability and activity depending on SIAH2 expression levels. Similarly, they suggest that the resistance to apoptosis induced by genotoxic agents in cells subjected to hypoxia is due, at least in

part, to the alteration of the DNA damage response-signaling pathway, which can be explained as a consequence of CHK2 depletion mediated by the expression of SIAH2.

6.2. CDC25A stability is regulated by the kinase DYRK2

6.2.1 CDC25A stability is proteasome and DYRK2 kinase activity dependent

In order to identify new DYRK2 substrates implicated in carcinogenesis, we performed a kinase array in which 1024 peptides were incubated with recombinant DYRK2 active protein *in vitro* (data not shown). The phosphatase CDC25A was found within the top 30 of the positive results in our screening. Given its essential role as a key regulator of the cell-cycle progression, we focused in studying in detail the effect of DYRK2 on CDC25A.

To study this effect, we first co-expressed CDC25A alone or in the presence of DYRK2 in HEK-293T cells. As shown in Figure 27A, protein levels of the phosphatase were reduced in the presence of increasing amounts of DYRK2. Similar results were obtained at endogenous levels when HeLa cells were transfected with increasing amounts of DYRK2, without affecting its mRNA expression (Figure 27B). Then we analyzed the effect of DYRK2 inhibition by siRNA on CDC25A expression (Figure 27C). DYRK2 depletion substantially increased CDC25A levels, indicating an inverse correlation between the expression of these two proteins. Taken together, these results suggest that DYRK2 regulates CDC25A turnover through a mechanism occurring at the protein level affecting its stability.

As CDC25A regulation by kinases is often accompanied by a proteasome dependent degradation (Busino *et al.*, 2004), we decided to analyze the role of this relevant mechanism in the case of DYRK2. We co-expressed CDC25A with increasing amounts of DYRK2 in the presence or absence of the proteasome inhibitor MG-132. As shown in Figure 28A, the reduction of the ectopic levels of CDC25A in response to DYRK2 was restored when cells were treated with MG-132.

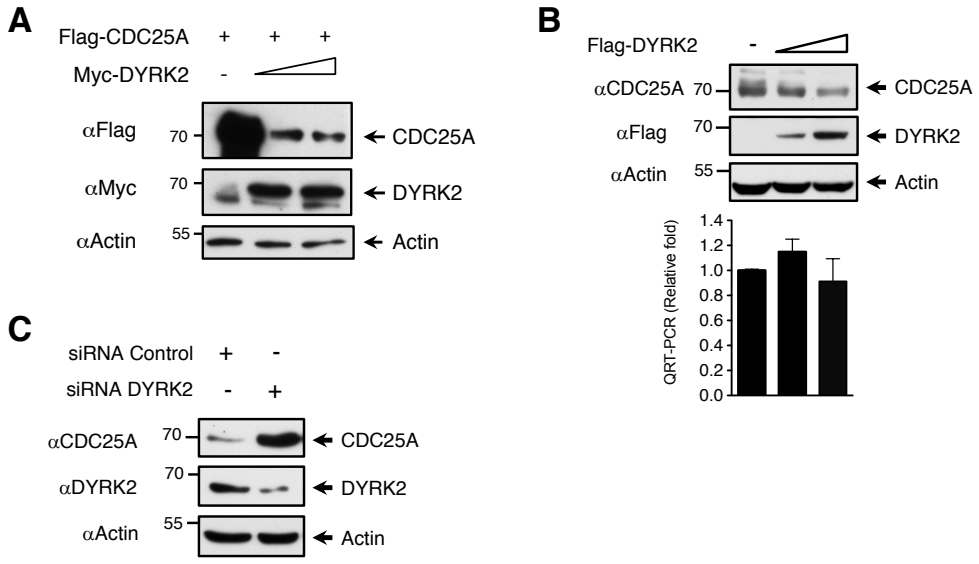

Figure 27. DYRK2 expression affects the stability of CDC25A. (A) HEK-293T cells were transfected with expression plasmids encoding Flag-CDC25A and Myc-DYRK2 as indicated. 48 h after transfection, cells were lysed and protein expression analyzed by immunoblot. We show a representative blot of three independent experiments. **(B)** HeLa cells were transfected to express increasing amounts of DYRK2, harvested and lysed. One fraction was used to analyze endogenous CDC25A protein levels while another aliquot was used to analyze CDC25A mRNA levels by qPCR. Data are mean ± SD of n = 3. We show a representative blot of three independent experiments. **(C)** HEK-293T cells were transfected with DYRK2 or scrambled (control) siRNAs, lysed after 4 days of culture and DYRK2 and CDC25A endogenous levels were analyzed by WB. We show a representative blot of three independent experiments.

Next, we decided to analyze the impact of DYRK2 kinase activity on CDC25A stability. DYRK2-wt overexpression was able to diminish the protein levels of CDC25A, an effect not observed with DYRK2 kinase dead mutant (KD) (Figure 28B). Moreover, we studied the effect of DYRK2 expression over CDC25A half-life. DYRK2-wt overexpression decreased the half-life of ectopic CDC25A, what did not occur in the presence of DYRK2-KD (Figure 29A). On the other hand, the half-life of endogenous CDC25A was prolonged under the knockdown of DYRK2 by specific siRNA (Figure 29B). Altogether, these results demonstrate that DYRK2 negatively regulates CDC25A expression through an ubiquitin/proteasome and DYRK2 kinase activity dependent process, as well as CDC25A basal levels are modulated according to DYRK2 expression.

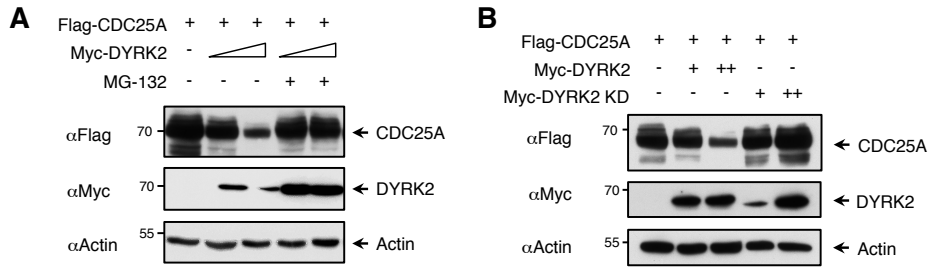

Figure 28. CDC25A degradation by DYRK2 is proteasome and DYRK2 kinase activity dependent. (A) HEK-293T cells were transfected with expression plasmids encoding Flag-CDC25A and Myc-DYRK2 and after 36 h the proteasome inhibitor MG-132 (10 μM) was added for another 12 h at the indicated points. Protein levels were analyzed by WB. **(B)** Cells were transfected with Flag-CDC25A together with Myc-DYRK2 wt (wild type) or KD (kinase death). Proteins were subjected to WB analysis with the indicated antibodies. We show a representative blot of three independent experiments.

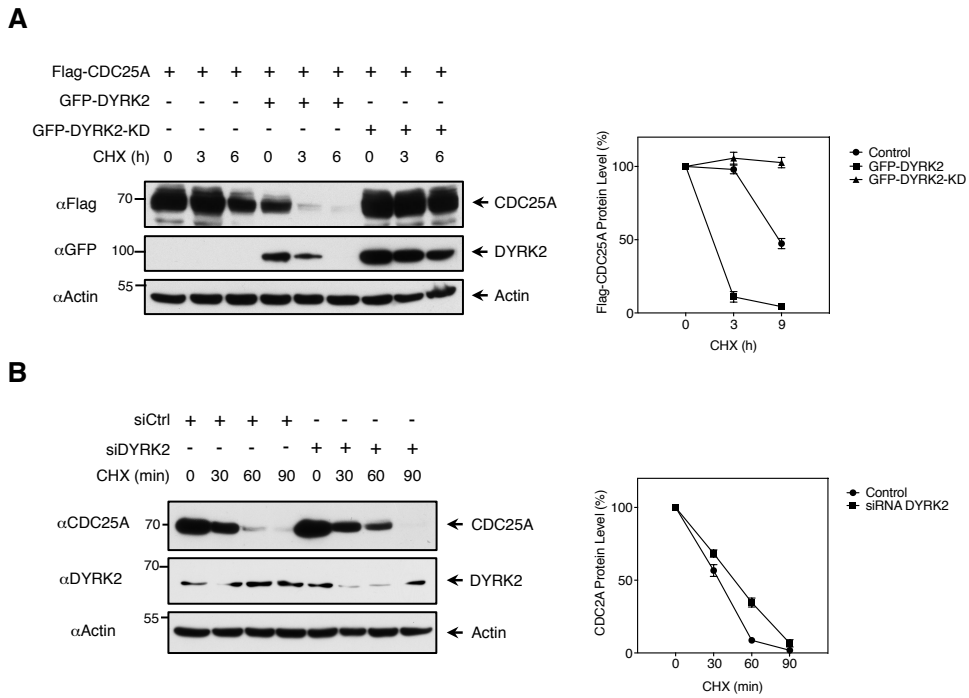

Figure 29. CDC25A half-life changes according to DYRK2 levels. (A) HEK-293T cells were transfected with CDC25A and DYRK2-wt or DYRK2-KD as indicated and after 36 h, treated with the protein synthesis inhibitor cycloheximide (CHX) (100 μg/ml) for 3 and 6 h. Cell lysates were analyzed by immunoblot using actin expression as the loading control. The graph represents the mean ± SD of band density from 3 different experiments. **(B)** Cells were transfected with either DYRK2 or scrambled (control) siRNAs and, after 4 days of culture, were incubated with CHX (100 μg/ml) during 30, 60 and 90 min. Cell lysates were analyzed by WB with the indicated antibodies. The graph represents the mean ± SD of band density from 3 different experiments.

6.2.2. CDC25A degradation is exclusive of DYRK subfamily

As mentioned before, DYRK2 belongs to the DYRK subfamily of protein kinases (Aranda et al., 2011). To test the ability of the rest of the members of this subfamily to affect CDC25A stability, HEK-293T cells were transfected to express CDC25A and the members of human DYRK subfamily (DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4). As shown in Figure 30, DYRK1B and DYRK2 overexpression resulted in a clear decrease of CDC25A protein levels, which was not observed in the case of DYRK1A, DYRK3 or DYRK4.

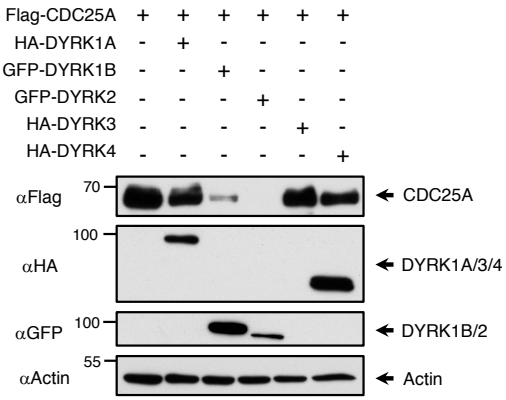

Figure 30. Effect of DYRK subfamily on CDC25A stability. HEK-293T cells were transfected to express the indicated combination of proteins. After 48 h, cells were lysed and protein expression was evaluated by immunoblot with the indicated antibodies. The positions and molecular weights (in kDa) are indicated. We show a representative blot of three independent experiments.

In the same sense, DYRK2 is highly related with HIPK2, other member of the DYRK family with which it shares some substrates like p53 *(Puca et al., 2010; Taira et al.*, 2007) or SIAH2 (Calzado *et al.*, 2009; Perez *et al.*, 2012). Therefore, we decided to evaluate the HIPK2 capacity to control CDC25A stability. Strikingly, HIPK2 overexpression increased ectopic CDC25A levels, being this effect HIPK2 kinase activity independent (Figure 31A). Next, we studied the role of HIPK2 on the effect of DNA damage over CDC25A and DYRK2 using HIPK2$^{-/-}$ and WT cells. Adriamycin treatment decreased substantially CDC25A levels and induced DYRK2 ones in both HIPK2$^{-/-}$ and WT cells (Figure 31B) without significant difference. Collectively, these

data show that CDC25A degradation is exclusive of DYRK subfamily, being DYRK2 the most effective in degrading the phosphatase among its members.

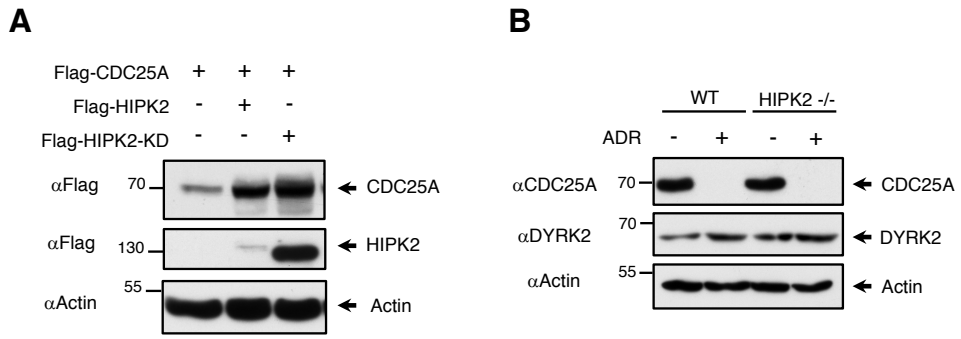

Figure 31. Role of HIPK2 on CDC25A degradation by DYRK2. (A) HEK-293T cells were transfected with the indicated plasmids. After 48 h, cells were lysed and proteins were subjected to WB analyses with the indicated antibodies. **(B)** HEK-293T WT and HEK-293T HIPK2$^{-/-}$ cells were treated with Adriamycin (ADR) (6 μM) during 24 h. Cells were lysed and protein expression was evaluated by WB using DYRK2 and CDC25A antibodies. We show a representative blot of three independent experiments.

6.2.3. DYRK2 phosphorylates CDC25A *in vitro*

To investigate whether DYRK2 directly phosphorylates CDC25A, we carried out *in vitro* phosphorylation analyses. First, we performed an *in vitro* kinase assay. Increasing amounts of recombinant DYRK2 resulted in the appearance of upper bands of recombinant CDC25A, corresponding to phosphorylated forms of the phosphatase as they were detected with a specific phospho-S/T-Pro antibody. Regarding DYRK2, the presence of CDC25A led to a faster migrated form of the kinase observed on SDS-PAGE (Figure 32A). To extend this finding further, recombinant CDC25A was incubated in the presence or absence of GST-DYRK2 forms (WT and KD). CDC25A phosphorylation was only detected by autoradiography when GST-DYRK2-wt was present (Figure 32B).

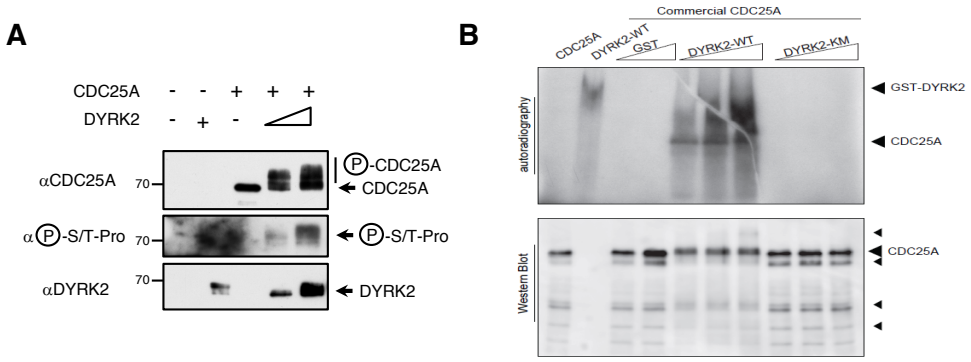

Figure 32. CDC25A is phosphorylated *in vitro* by DYRK2. (A) *In vitro* kinase assay was performed with active DYRK2 and CDC25A recombinant proteins. CDC25A phosphorylation was determined with phospho-S/T-Pro antibody, while total protein levels of DYRK2 and CDC25A were recognized by its specific antibodies. **(B)** Active CDC25A recombinant protein was incubated with GST-DYRK2-wt, GST-DYRK2-KD or GST alone. Phosphorylation was determined by autoradiography, while protein levels of CDC25A were detected by WB.

It is widely described that CDC25A degradation by the proteasome is mainly regulated by $SCF^{\beta TRCP}$ and APC/C ubiquitin-ligase complexes (Busino *et al.*, 2004; Busino *et al.*, 2003; Donzelli et al., 2002), which need to recognize specific residues phosphorylated within the DSG motif or bind to KEN motif, respectively, in order to accomplish the degradation of the phosphatase. Given that DYRK2 promotes CDC25A degradation by the proteasome (Figure 28A), we generated CDC25A DSG and KEN mutants (Figure 33A) to study whether these motifs were implicated in the process. Particularly, within DSG motif, Ser88, but not Ser82, is followed by a proline. Bearing in mind that DYRK2 has a preference for phosphorylating serines followed by proline (DYRK2 consensus: R/K X X (X) **S/**T P/V), we mutated Ser88 to alanine to generate the DSG mutant. HEK-293T cells were transfected with CDC25A WT or mutants forms together or not with DYRK2. Surprisingly, DYRK2 degraded similarly either CDC25A WT or CDC25A mutants (Figure 33B). All these results clearly indicate that DYRK2 directly phosphorylates CDC25A *in vitro*, although DSG or KEN motifs are not involved in the CDC25A degradation by the proteasome mediated by DYRK2.

Since DYRK2 directly phosphorylates CDC25A, it is reasonable to consider that both proteins are interaction partners. To test this hypothesis, we performed different methodological approximations including co-immunoprecipitation assays but,

unfortunately, we were unable to pull down the phosphatase together with DYRK2 or vice versa (data not shown).

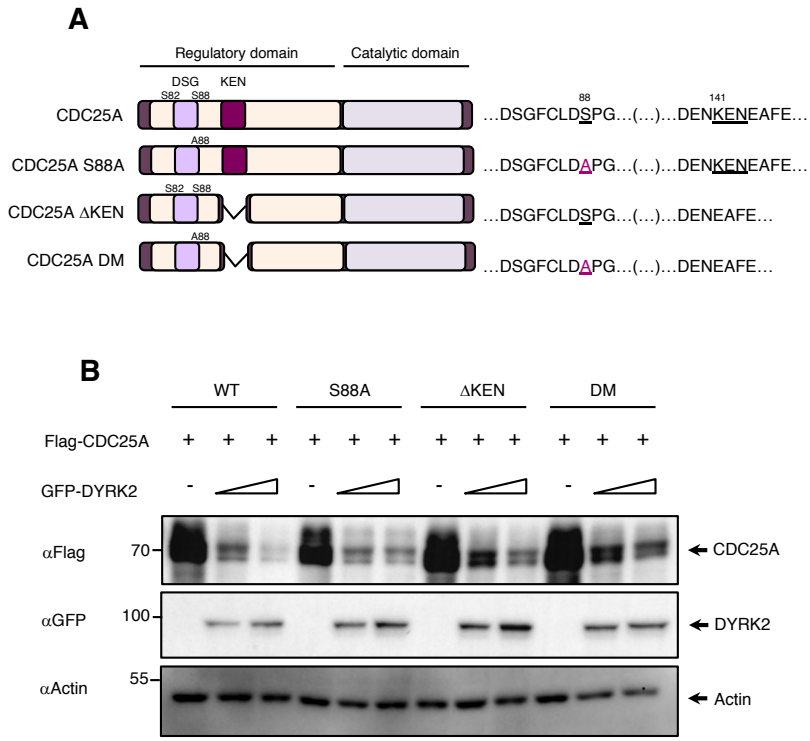

Figure 33. DSG or KEN motifs are not involved in the CDC25A degradation by DYRK2. (A) Schematic representation of CDC25A-wt and its mutants (S88 →A88 (S88A) within DSG motif; KEN motif deletion (ΔKEN); double mutant (S88A + ΔKEN)). **(B)** HEK-293T cells were transfected with CDC25A-wt or CDC25A mutants together or not with GFP-DYRK2. Cell lysates were analyzed by WB with the indicated antibodies. We show a representative blot of three independent experiments.

6.2.4. DYRK2-CDC25A protein levels correlation under different stimuli

Given the fact that we were unable to detect a direct DYRK2-CDC25A complex, we could not rule out the possibility of the existence of intermediate partners between both proteins. We examined whether proteins belonging to the ATM/ATR DNA damage pathway or some phosphatases were playing a role in the CDC25A regulation by DYRK2. No differences were observed on CDC25A degradation performed by DYRK2 when cells were incubated with Caffeine, an ATM/ATR inhibitor (Figure 34A), or

Calyculin A, a phosphatase inhibitor (Figure 34B) compared with control ones. These data indicate that CDC25A degradation by DYRK2 is ATM/ATR and phosphatases independent.

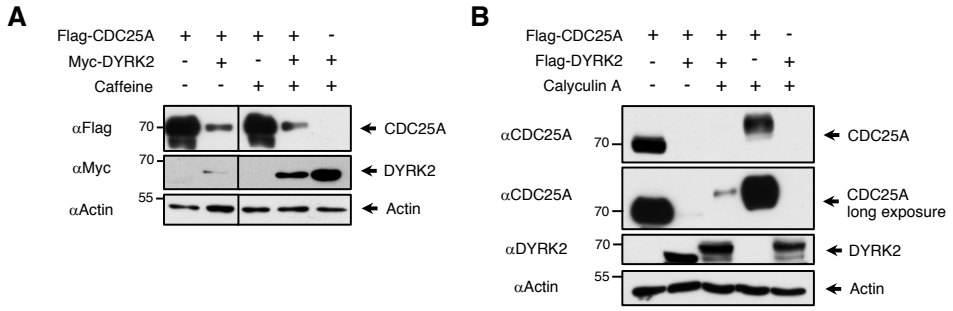

Figure 34. DYRK2 effect over CDC25A is phosphatase and ATM/ATR independent. **(A)** HEK-293T cells were transfected to express the indicated combination of proteins. After 48 h, cells were incubated with Caffeine (3 mM) during 3 h as shown. Cell lysates were analyzed by WB with the indicated antibodies. We show a representative blot of three independent experiments. **(B)** HEK-293T cells were co-transfected to express CDC25A and DYRK2 at the indicated points. 48 h after transfection, Calyculin A (100 nM) was added for another 30 min as indicated. Cells were lysed and protein expression analyzed by immunoblotting.

DYRK2 protein expression variations under different stimuli have been reported previously (Guo *et al.*, 2016; Zhang *et al.*, 2016). To evaluate the behavior of the phosphatase under these conditions, several cell lines were subjected to serum starvation or DNA damage. When cells were serum starved (synchronized at G1) and then released, it was observed a clear relation between DYRK2 and CDC25A protein levels H727 cells (Figure 35A). Similar results were obtained after double thymidine synchronization of HeLa cells in concrete phases of the cell cycle, specifically at G2/M (Figure 35B). Furthermore, ADR treatment increased DYRK2 expression levels in HEK-293T cells, what resulted in a reduction of CDC25A that was restored in part by DYRK2 specific siRNA silencing (Figure 35C). In MOR cells, an ADR dose dependent DYRK2 stabilization accompanied by a CDC25A inhibition was detected (Figure 35D). These experiments demonstrate that an inverse correlation exists between both proteins in response to different stimuli.

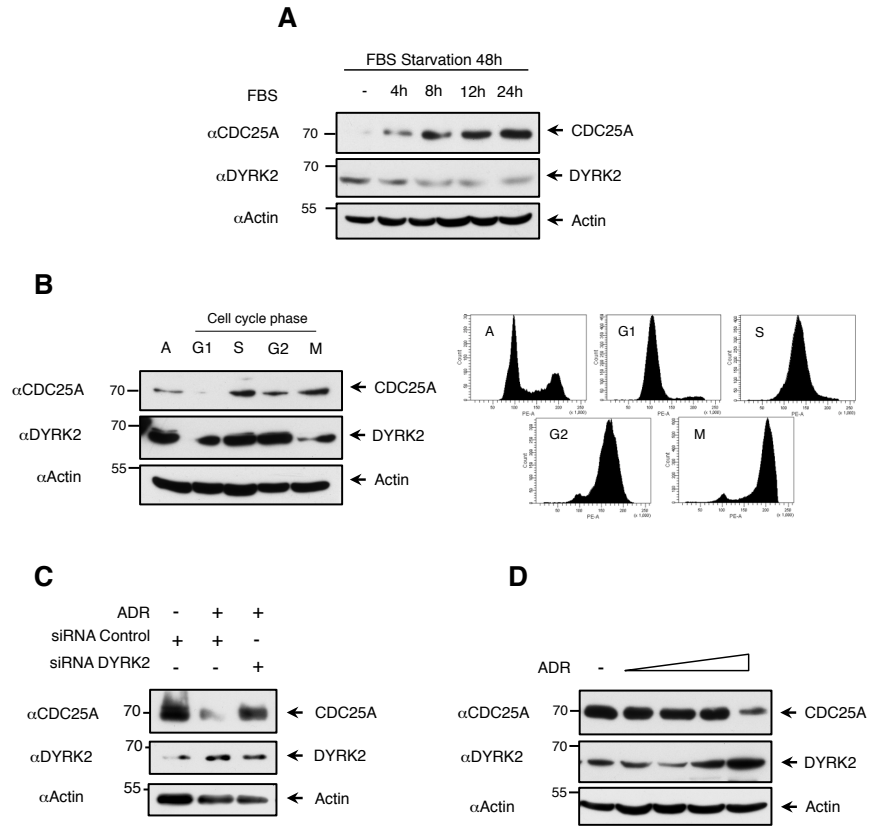

Figure 35. DYRK2-CDC25A protein levels correlation under different stimuli. (A) H727 cells were serum starved for 48 h (synchronized at G1) and then released (serum stimulation) for 0, 4, 8, 12, or 24 h (entered into the cell cycle). Protein fraction was subjected to WB analyses with the indicated antibodies. **(B)** HeLa cells were synchronized by double-thymidine block, then released into fresh media and harvested after 0 (G1 fraction), 3.5 (S fraction), 6 (G2 fraction) and 10 h (M fraction); A = asyncronized control cells. Cell cycle was analyzed by PI staining and flow cytometry (left panel). The expression of DYRK2 and CDC25A proteins evaluated in parallel by WB (right panel). **(C)** HEK-293T cells were transfected with DYRK2 or scrambled (control) siRNAs and after 3 days incubated with ADR (6 μM) for another 24 h. Cells were lysed and protein expression analyzed by immunoblot. **(D)** MOR cells were stimulated with increasing ADR concentrations (1, 2, 4 and 6 μM) during 24 h. Cell lysates were analyzed by WB with the indicated antibodies. We show a representative blot of three independent experiments.

6.2.5. Correlation of DYRK2 and CDC25A in human lung cancer cells (HLCC)

It has been previously described an important role of DYRK2 in pulmonary carcinogenesis (Miller et al., 2003), together with increased expression of CDC25A (Boutros *et al.*, 2007). Based on this and other evidences, we decided to evaluate the

relation between CDC25A and DYRK2 in different human lung cancer cell lines. First, we studied endogenous levels of both proteins in the BEAS-2B model, in which human bronchial epithelial cells undergo squamous differentiation in response to serum (Figure 36A). This model helps us to better understand the carcinogenesis process in lung cancer. Corroborating previous results, this model showed us an inverse correlation between DYRK2 and CDC25A. Moreover, the more differentiated the cells are, a higher phosphatase expression and a lower kinase expression was observed (Figure 36B). This correlation was also confirmed at endogenous levels in other lung cancer cells, such as A549 and H727 (Figure 36B).

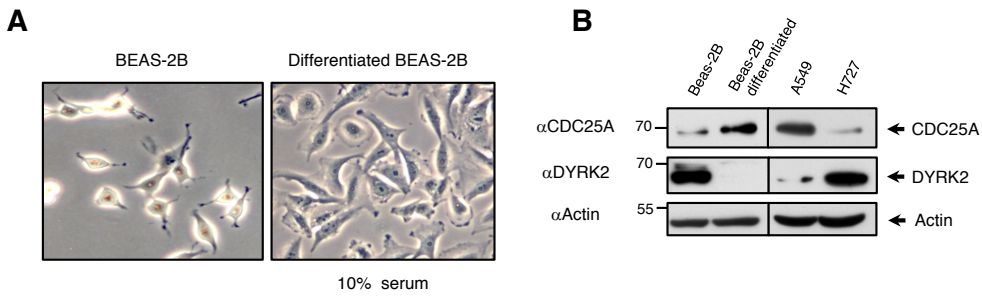

Figure 36. Expression and correlation between DYRK2 and CDC25A in HLCC. (A) Illustrative images of BEAS-2B differentiation model, in which human bronchial epithelial cells (BEAS-2B; left panel) undergo squamous differentiation in response to serum (right panel). **(B)** Different HLCC were lysed and the protein fraction was analyzed by WB with specific DYRK2 and CDC25A antibodies. We show a representative blot of three independent experiments.

Next, differentiated BEAS-2B cells were transfected to express DYRK2-wt or DYRK2-KD (in the presence or absence of MG-132). Ectopic and endogenous CDC25A levels were analyzed under these conditions. The results demonstrated that DYRK2 overexpression degraded CDC25A at both ectopic (Figure 37A) and endogenous levels (Figures 37B). Likewise, ectopic CDC25A degradation by DYRK2 was also observed in H727 cells (Figure 37C). Once again, this degradation was noticed to be proteasome and DYRK2 kinase activity dependent. Taken together, these results confirm our biochemical model of CDC25A regulation by DYRK2 in lung cancer cell lines.

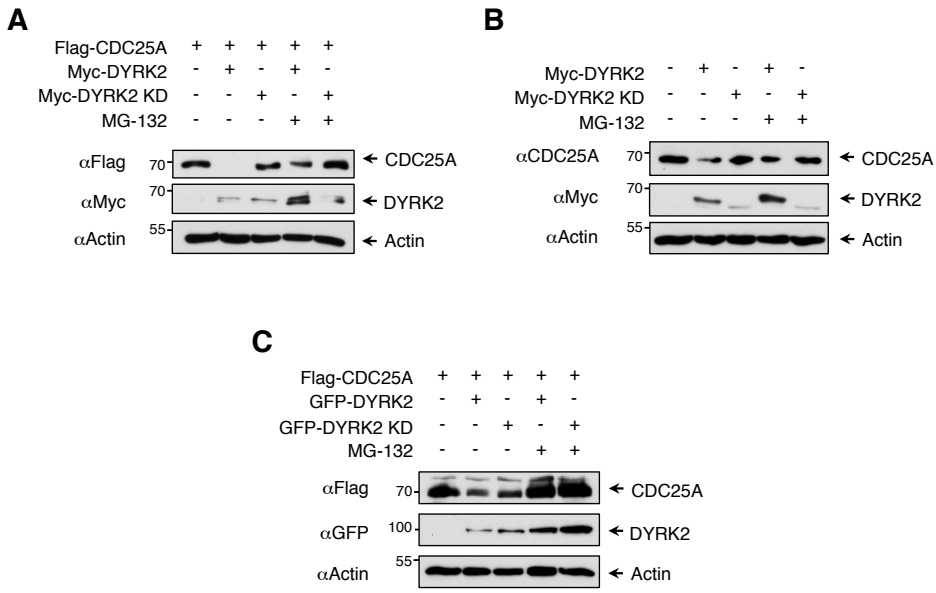

Figure 37. CDC25A degradation by DYRK2 in HLCC. Differentiated BEAS-2B **(A)** and H727 cells **(C)** were co-transfected to express CDC25A together with DYRK2-wt or DYRK2-KD (in the presence or absence of MG-132). Cell lysates were analyzed by WB with the indicated antibodies. **(B)** Differentiated BEAS-2B cells were transfected with Myc-DYRK2-wt or Myc-DYRK2-KD (in the presence or absence of MG-132). Cells were lysed and protein expression analyzed by immunoblot. We show a representative blot of three independent experiments.

6.2.6. DYRK2 and CDC25A expression in human lung cancer tissue

We have previously demonstrate the significant decrease in DYRK2 expression in samples of patients with lung cancer (adenocarcinoma and squamous) compared to their corresponding healthy ones by immunohistochemistry (IHC) (Moreno *et al.*, 2015). We then decided to correlate this outcome with published CDC25A expression data in human lung cancer tissues. In contrast to DYRK2, CDC25A expression is increased in adenocarcinoma and squamous lung cancer tissues (Protein Atlas) (Figure 38A). Moreover, comparison of reported DYRK2 and CDC25A protein expression levels in a variety of tumors reveals an inverse correlation between them, with the exception of melanoma and endometrial, testis and prostate cancers (Figure 38B). Once more, it is observed an inverse correlation of the protein expression of DYRK2 and CDC25A in tumors, including lung cancer, that confirm and validate our previous results.

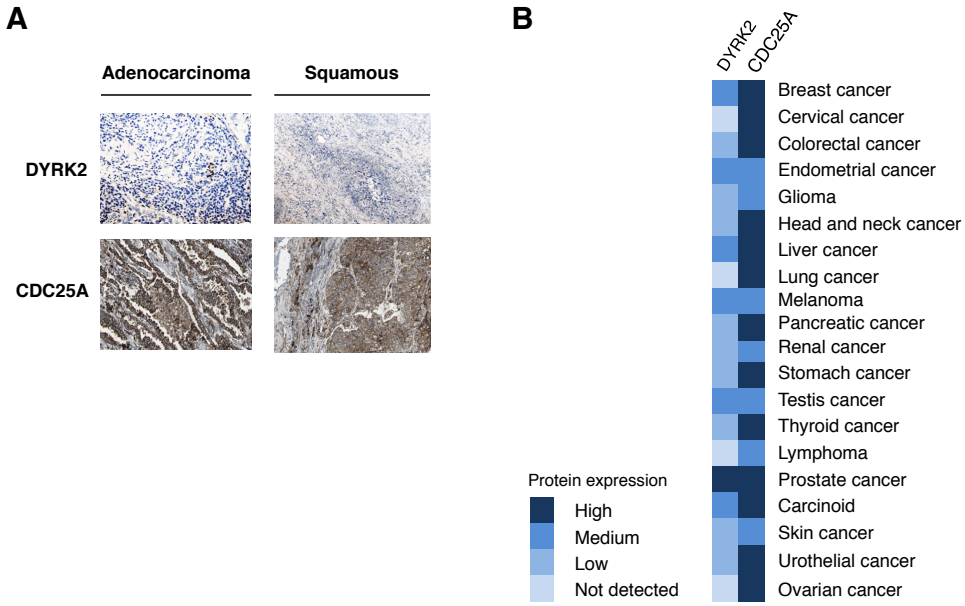

Figure 38. Correlation of DYRK2 and CDC25A expression in human lung cancer tissue. (A) Representative images of adenocarcinoma or squamous cell carcinoma stained with DYRK2 antibody (*adapted from Moreno et al, 2015*) or CDC25A antibody (*adapted from Protein Atlas*) (x100). **(B)** Comparative study of reported DYRK2 and CDC25A protein expression levels in a wide range of tumors (*adapted from Protein Atlas*).

Discussion

7.1. CHK2 stability is regulated by the E3 ubiquitin ligase SIAH2

The ubiquitin-proteasome pathway is critical for the expression and activity control of proteins implicated in cell cycle control and DNA damage response (Kirkin & Dikic, 2011; Liu *et al.*, 2014). CHK2 is a stable protein with a 6-hour half-life whose regulation and degradation mechanisms in the absence of DNA damage are not completely known (Lukas *et al.*, 2001; Zannini *et al.*, 2014). Although the phosphorylation levels and the action of several phosphatases are the most relevant mechanism to keep CHK2 inactive, (Carlessi *et al.*, 2010; Freeman *et al.*, 2010; Fujimoto *et al.*, 2006) different evidences suggest the role of ubiquitination to control the stability of this kinase. Firstly, the capacity of Ub E3 ligase Mdm2 and acetyltransferase PCAF to increase CHK2 ubiquitination has been described, but in an independent way to their E3 ligase activities (Kass *et al.*, 2009). Similarly, the role of EDD in CHK2 activation has been reported, although its ability to mediate CHK2 ubiquitination has not been demostrated yet (Munoz *et al.*, 2007). Recently, the ability of the E3 ligase RNF8 in human, and PIRH2 in mice to ubiquitylate and degrade CHK2 has been characterized (Bohgaki *et al.*, 2013; Feng & Chen, 2012). It should be underlined that, whereas these two E3 ligases mediate degradation in response to DNA damage, our results suggest that SIAH2 regulates CHK2 basal expression.

In the present work we describe the capacity of CHK2 to directly interact *in vivo* with SIAH2, mainly colocalizing in the nucleus. Through different experimental approaches, we prove that SIAH2 mediates CHK2 ubiquitination and proteosomal degradation. SIAH2 depletion increases CHK2 levels, thereby supporting our observation that SIAH2-deficient cells show high CHK2 levels. Phosphorylation in S456, which has been proved to be critical for CHK2 ubiquitination, is not here relevant for CHK2 degradation (Kass *et al.*, 2007). Similarly, our results show that SIAH2-mediated CHK2 degradation is independent of both phosphorylation in T68 and its kinase activity. Likewise, DNA damage alters the interaction between both proteins, thus decreasing SIAH2-mediated CHK2 levels and not affecting the endogenous levels of CHK2 T68 or S516. These results suggest that SIAH2 interacts and regulates a pool of CHK2 in the absence of stimuli. In response to DNA damage, a CHK2 fraction would

split from SIAH2, thus avoiding its degradation and allowing its stabilization. Although the exact mechanism underlying the separation of SIAH2 from CHK2 after DNA damage is unknown, it seems reasonable to think that it could be the result of post-transductional modifications suffered by any of the two proteins in response to this stimulus. The absence of a complete dissociation of both proteins in response to DNA damage observed in our results could explain the existence of different CHK2 fractions controlled by SIAH2 contributing to the cell cycle control (Figure 27).

The regulation of ubiquitin ligases activity is critical for the control of its substrates. Different control mechanisms have been described for SIAH family, amongst which the role of phosphorylation stands out (Knauer *et al.*, 2015). Others and we have reported that SIAH2 phosphorylation affects its activity, and three kinases with SIAH2 as substrate have been described so far (Calzado *et al.*, 2009; Khurana *et al.*, 2006; Perez *et al.*, 2012). In this work we also highlight CHK2 capacity to phosphorylate SIAH2 in at least three residues, not affecting its activity on this kinase, but decreasing its ability to degrade other substrates. These results are consistent with previous reports describing how SIAH2 phosphorylation modulates its activity specifically on certain substrates (House *et al.*, 2009; Perez *et al.*, 2012; Winter *et al.*, 2008). Similarly, our results show how ETP stimulation significantly reduces CHK2-mediated SIAH2 phosphorylation, in agreement with the loss of interaction between these two proteins. As a consequence, the activity of SIAH2 on some of its substrates in response to DNA damage would be modified. The changes observed in some SIAH2 substrates in response to DNA damage such as DYRK2 are in accord with this hypothesis (Taira *et al.*, 2007; Taira *et al.*, 2010).

From the functional point of view, CHK2 plays a relevant role in the cell cycle control in response to DNA damage, inhibiting CDC25C phosphatase through Ser216 phosphorylation and avoiding mitosis (Bulavin *et al.*, 2003; Zannini *et al.*, 2014). Our results demostrate how SIAH2 expression resulted in a clear reversion of cellular arrest induced by ETP stimulation. Besides, we demonstrate that the loss of SIAH2-mediated CHK2 partially prevented ETP-induced apoptosis en HeLa cells, which was recovered by returning CHK2 protein expression to endogenous levels. These data prove that

SIAH2 expression affects cell cycle activation checkpoints through the control of CHK2 levels.

Finally, many studies support the concept that hypoxia is directly implicated in tumor progression and metastasis, mediating apoptosis resistance and reducing the ability to repair DNA (Bristow & Hill, 2008; Wilson & Hay, 2011). Different works have reported how intratumoral hypoxia significantly modifies the action of some drugs and promotes metastasis (Frankenberg-Schwager *et al.*, 1991; Wilson & Hay, 2011). Similarly, cells subjected to hypoxia present a lower capacity to repair DNA and, as a consequence, they become more resistant to chemotherapy (Blais *et al.*, 2006; Terris *et al.*, 2002). Different clinical trials that attempted to diminish tumor hypoxia have significantly improved cell sensibility to radiotherapy and chemotherapy, which resulted in a higher rate of survival (Overgaard, 2007). In general, the concept that hypoxia increases cell resistance to apoptosis through different mechanisms is consistent with our results. SIAH2 has the capacity to regulate HIF-1α levels, and thus the physiological response to hypoxia (Nakayama *et al.*, 2004). We observed that hypoxia decreases CHK2 levels, parallel to an increase of both SIAH2 and cell resistance to apoptosis induced by ETP. We have also observed how SIAH2 expression increases cell viability in response to cytotoxic drugs such as 5-FU or Oxaliplatin. These data are equally supported, at least partially, by previous results which also showed lower CHK2 levels under hypoxia in HeLa and MCF-7 cells (Gibson *et al.*, 2005). Altogether, this finding suggests a new action mechanism by which hypoxia may alter DNA damage-response pathway via SIAH2-CHK2 modulation.

In summary, our results indicate that E3 Ubiquitin ligase SIAH2 regulates CHK2 basal turnover. Additionally, CHK2 presents the ability to phosphorylate SIAH2, affecting its activity on certain substrates. In response to DNA damage, the interaction between both proteins is altered, which favors CHK2 stabilization. We propose that this new CHK2 regulation mechanism has an influence on cell cycle control and on the ability of hypoxia to alter DNA damage-response pathway in cancer cells. Further studies of the relationship of these two proteins in tumors are needed to support this hypothesis.

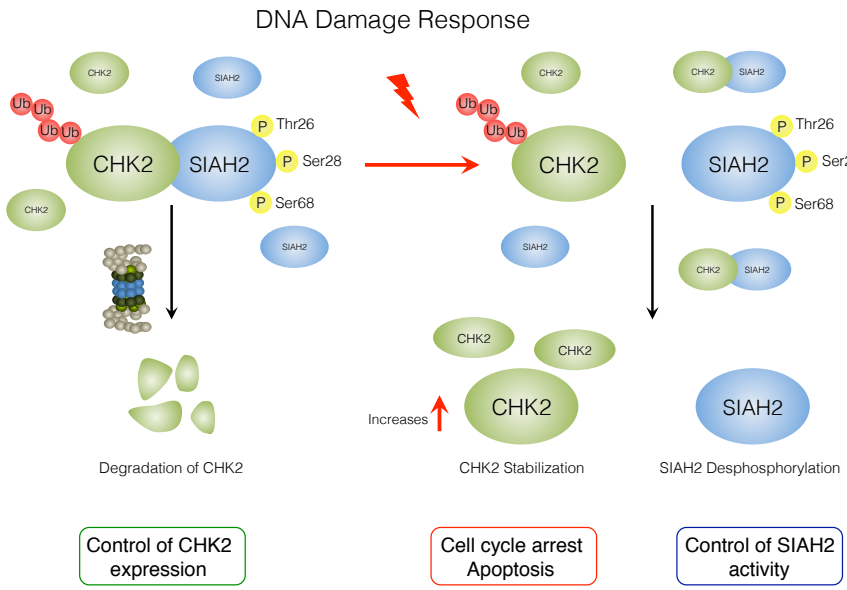

Figure 39. Schematic model for the ability of SIAH2 to regulate CHK2 by ubiquitination. Under normal conditions SIAH2 is able to interact and modulate the basal turnover of CHK2 by ubiquitination and proteasome degradation. Simultaneously, CHK2 is able to phosphorylate SIAH2 in at least three residues (Thr26, Ser28 and Ser68). The DNA-damage response causes alteration in the protein-protein interaction, promoting CHK2 stabilization and SIAH2 activity modification.

7.2. CDC25A stability is regulated by the kinase DYRK2

Due to its major function as key controller of normal cell division and mediator of the checkpoint response to DNA damage, CDC25A regulation results essential for the proper cell division. One of the most studied post-translational mechanisms of CDC25A regulation is its degradation through the ubiquitin–proteasome pathway (Busino *et al.*, 2004), mainly mediated by $SCF^{\beta TRCP}$ and APC/CCdh1 ubiquitin-ligase complexes (Busino *et al.*, 2003; Donzelli *et al.*, 2002). For that purpose, in the majority of cases, CDC25A is required to be previously phosphorylated in specific residues. The kinases described to date able to achieve that task are CHK1 and CHK2, p38, CK1A and NEK11 (Boutros *et al.*, 2007; Busino *et al.*, 2004; Honaker & Piwnica-Worms, 2010; Melixetian *et al.*, 2009), that seems to be a reduced number of CDC25A regulators given the relevant function of the phosphatase, what in turn indicates that more kinases could be involved in the mechanism. Here we describe a new kinase able to mediate

post-translational regulation of CDC25A. DYRK2 negatively regulates CDC25A expression through an ubiquitin/proteasome and DYRK2 kinase activity dependent process, resulting in a significant reduction of its half-life. At first instance our results indicate that CDC25A degradation by DYRK2 is ATM/ATR pathway independent, suggesting that DYRK2 regulates the basal turnover of CDC25A, and therefore being involved in the normal control of cell cycle but not in the regulation of the phosphatase in response to DNA damage. Nevertheless, we have observed that the reduction of CDC25A under the DNA damage agent ADR is restored in part by DYRK2 specific siRNA silencing. Likewise, the ADR dose dependent DYRK2 stabilization is accompanied by a CDC25A inhibition. Thus, we can not rule out that DYRK2 could be also taking part in CDC25A regulation under DNA damage. These results may arise the possibility of a dual role of DYRK2 in regulating CDC25A expression, both under normal conditions and under DNA damage, as it occurs in the case of CHK1, CHK2 or p38 (Boutros *et al.*, 2006; Busino *et al.*, 2004).

Although DYRK2 is the most effective in degrading CDC25A among the members of DYRK subfamily, we observed that the presence of DYRK1B also had a role regarding the phosphatase degradation. Besides DYRK1B and DYRK2 belong to different groups within the DYRK subfamily (class I and class II, respectively), it appears that they have similar functions concerning other substrates, as it has also been proved by us (Perez *et al.*, 2012) and various colleagues (unpublished data). On the other hand, DYRK2 shares some substrates with its highly related HIPK2 kinase, such as p53 (Puca *et al.*, 2010; Taira *et al.*, 2007) or SIAH2 (Calzado *et al.*, 2009; Perez *et al.*, 2012). In the case of CDC25A, HIPK2 does not degrade the phosphatase but increases its protein levels (both wt and KD forms of the kinase), without interfering with CDC25A degradation by DYRK2.

In order to go further in examining the possible sites for DYRK2 phosphorylation on the phosphatase, we generated CDC25A DSG and KEN motifs mutants, given that they are needed for $SCF^{\beta TRCP}$ and APC/C mediated-CDC25A proteasome degradation, respectively (Busino *et al.*, 2004; Busino *et al.*, 2003; Donzelli *et al.*, 2002). Furthermore, a recent study revealed that $SCF^{\beta TRCP}$ is responsible of

DYRK1A proteasome degradation, what results essential for cell cycle progression (Liu *et al*., 2016). The fact that DYRK1A is related to $SCF^{\beta TRC}$, reinforced our hypothesis of the implication of this complex in DYRK2-CDC25A regulation. However and unexpected for us, DYRK2 expression degraded similarly either CDC25A WT or CDC25A DSG and KEN mutants, indicating that DYRK2-mediated proteasome degradation of the phosphatase may not be occurring via the classical ubiquitin-proteasome pathways described for it. On the other hand, the process could be taking place via an alternative ubiquitin pathway, like the DYRK2-related SCF^{Fbw7} or EDVP (Jung *et al*., 2013; Maddika & Chen, 2009) or the CDC25A-related Cul4B-$DDB1^{DCAF8}$ (Wu *et al*., 2016), or even unidentified ones, thus opening up a new horizon in this field.

It is a fact that fluctuations in CDC25A protein levels exist during the cell cycle, being a result from an exhaustive regulation by periodic synthesis and by ubiquitin mediated proteolysis. At the end of mitosis and during G1, CDC25A protein levels are kept low by APC/CCdh1, after what they are increased because of the requirement of the phosphatase for G1/S transition. During S and G2 phases, CDC25A levels are regulated by $SCF^{\beta TCRP}$-dependent turnover and finally, over mitosis CDC25A is stabilized by Cyclin B/CDK1 phosphorylation on Ser18 and S116, which acts as a positive-feedback mechanism for Cyclin B/CDK1 activation, and therefore, allowing G2-M transition of the cells (Hayes & Harper, 2010). Our results regarding CDC25A expression levels that arise from HeLa cell cycle analyses are consistent with these previous published data. In terms of DYRK2 expression during cell cycle, it has been reported its upregulation at the start of S phase that persists, but decreasing slightly, throughout G2 and M phases in HaCat cells (Guo & Dixon, 2016; Guo *et al*., 2016). Our data is in concordance with that observation, since we also observe in HeLa cells a markedly overexpression of DYRK2 in S and G2 phases that is not maintained at those levels during mitosis. Moreover, these cell cycle analyses show us a clear relation between DYRK2 and CDC25A expression levels at two specific phases of the cell cycle: G2 and M. This outcome suggests a probably specific role of DYRK2 over CDC25A during G2 and/or M phases, could being influenced by DYRK2 expression levels and/or by its activity. Further studies are needed to achieve a complete understanding of this mechanism.

Over the last decade, it has been enhanced the evidence of the engagement of DYRK2 in proper control for cell division, through the phosphorylation and consequent degradation of diverse substrates including c-Jun/c-Myc, Tert and Kataninp60 (Nihira & Yoshida, 2015). Most importantly, DYRK2 functions as a priming kinase for GSK3 and the SCF^{Fbw7} complex in G1 phase, because c-Jun and c-Myc degradation is essential for G1/S transition (Taira *et al.*, 2012). However, DYRK2 binds to the EDVP complex and triggers proteasomal degradation of TERT (Jung *et al.*, 2013) and Katanin p60 (Maddika & Chen, 2009) in G2/M phase. More recently it has been described that DYRK2 phosphorylates Rpt3 (proteasome subunit) at Thr25 in a cell cycle-dependent manner, mainly during S and G2/M phases (Guo & Dixon, 2016). Because dysregulation of cell cycle progression leads to tumorigenesis, DYRK2 might have a tumor suppressive role in cancer cells. According to these data, here we have proved that DYRK2 directly phosphorylates the checkpoint phosphatase CDC25A *in vitro,* increasing this way the list of DYRK2 substrates that are involved in the control of the cell cycle. As mentioned above, further research is needed to elucidate the role of DYRK2 over CDC25A in the different phases of the cell cycle. While it is true that we were not able to pull down both proteins together, it does not mean that they are not interaction partners. Sometimes the interaction between kinase and substrate is real ephemeral and short lived, thus hampering its detection by experimental approaches. Moreover, we need to keep in mind that DYRK2 could be degrading CDC25A as a part of a complex (such as previous mentioned SCF^{Fbw7} or EDVP), what may difficult this observation as well.

Despite there are some studies that have reported a high DYRK2 expression in esophagus and lung cancers and neuroblastoma (Miller *et al.*, 2003; Park *et al.*, 2002; Varjosalo *et al.*, 2008), in general, it has been described a reduced or abolished DYRK2 expression in multiple human tumor tissues, including kidney, lung, breast, colon, prostate, esophagus, bladder and hepatocarcinoma. In most of them the low DYRK2 expression correlates with shorter survival (Yamashita *et al.*, 2009), invasiveness (Taira *et al.*, 2012), cancer recurrence (Enomoto *et al.*, 2014) or poor prognosis (Yan *et al.*, 2016). Moreover, DYRK2 has been also proposed as potential prognostic marker for cancer and metastasis (Nomura *et al.*, 2015) and as predictive marker for clinical

responses to cancer treatment (Mimoto *et al.*, 2017). These data shows that a lack of DYRK2 expression leads to cancer development in a wide range of tissues, supporting the role of DYRK2 as a tumor suppressor. Therefore, up-regulation of DYRK2 expression or activity in that tissues might be a useful strategy for cancer prevention (Nihira & Yoshida, 2015).

In contrast with the above, CDC25A is considered an oncogene since it is highly overexpressed in most cancer tissues, where accelerates the G1/S and G2/M transitions, leading to genomic instability and being responsible for tumorigenesis promotion. CDC25A has been reported to be overexpressed in various human cancers, including breast, colorectal, lung pancreas, hepatocellular, thyroid and non-Hodgkin lymphoma among others, being highly associated a shorter disease-free survival, malignancy and poor prognosis in cancer patients (Boutros *et al.*, 2007; Kristjansdottir & Rudolph, 2004). Consequently, CDC25A inhibition is considered as a potential therapeutic target against cancer, but unfortunately to date there is no specific inhibitors of CDC25A, since the ones available show cross-reaction with the three CDC25 isoforms (Brenner *et al.*, 2014).

In summary, in the present work we describe the capacity of DYRK2 to negatively regulates the checkpoint phosphatase CDC25A expression through an ubiquitin/proteasome and kinase activity dependent process. As well, we demonstrate that DYRK2 is capable to directly phosphorylate the phosphatase *in vitro.* Different stimuli, such as DNA damage or serum starvation, result in an inverse correlation of DYRK2 and CDC25A expression, which is also observed at endogenous levels in human lung cancer cell lines and human lung cancer tissues (Figure 40).

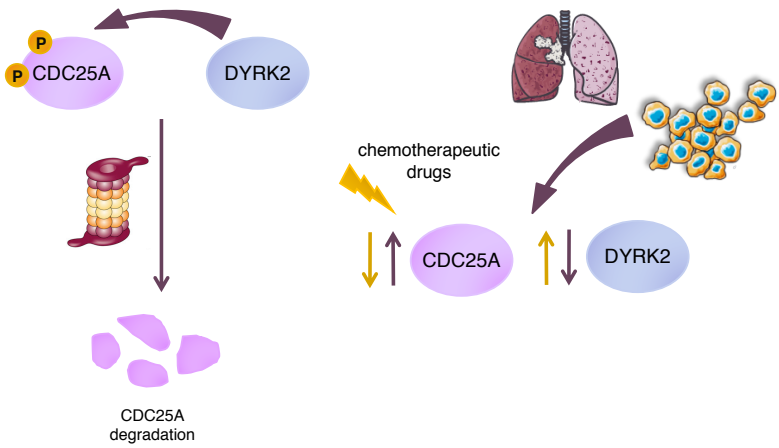

Figure 40. Schematic model for the ability of DYRK2 to regulate CDC25A. DYRK2 is able to modulate the basal turnover of CDC25A by phosphorylation and proteasome degradation (left panel). We report the existence of an inverse correlation of DYRK2 and CDC25A expression levels in lung cancer cell lines as well as in human tumors of the lung (right panel). Chemotherapeutics drugs switch this correlation the other way around.

Taken together, our findings report new substrates (SIAH2 and CDC25A) for two relevant kinases involved in the DNA Damage Response pathway (CHK2 and DYRK2), helping to elucidate the molecular mechanisms through which this pathway operates. A deeper understanding of the regulation mechanisms of these relevant kinases will extend the knowledge about the molecular biology of cancer as well as provide new therapeutic opportunities for cancer treatment. Additionally, examining the collateral genomic damage caused by DNA repair deficiency could provide biomarkers to aid in the selection of cancer therapy, not only predicting the sensitivity of DNA damaging agents that maximize cytotoxicity for cancer cells, but also determining the proper dosing of DNA damage drugs to minimize the side effect for normal cells. This way, personalized cancer treatments would be achieved.

Conclusions

1. It exists a direct physical interaction between CHK2 and the E3 Ubiquitin ligase SIAH2, being several domains responsible for binding in both proteins. CHK2-SIAH2 complex is partially disrupted in response to DNA damage without impairing its subcellular co-localization, but favoring CHK2 stabilization.

2. CHK2 presents the ability to directly phosphorylate SIAH2 at a minimum of three residues, without affecting its capacity to degrade this kinase but modulating its activity on other substrates such as PHD3, DYRK2 and HIPK2.

3. SIAH2, in turn, is capable to regulate the basal turnover of CHK2, through a SIAH2-mediated ubiquitination and proteasomal degradation occurring at the protein level, affecting its stability and activity.

4. CHK2 regulation by SIAH2 has an influence on cell cycle control and on the ability of hypoxia to alter DNA damage-response pathway in cancer cells.

5. DYRK2 negatively regulates the checkpoint phosphatase CDC25A expression through an ubiquitin/proteasome and DYRK2 kinase activity dependent process, affecting its half-life.

6. DYRK2 is the most effective in degrading the phosphatase among the members of its subfamily. CDC25A degradation by DYRK2 is not influence by HIPK2.

7. DYRK2 directly phosphorylates CDC25A *in vitro*. DSG or KEN motifs of CDC25A are not involved in the proteasome degradation mediated by DYRK2.

8. Different stimuli, such as DNA damage or serum starvation, result in an inverse correlation of DYRK2 and CDC25A expression, which is also observed at endogenous levels in human lung cancer cell lines and human lung cancer tissues.

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Annexes

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ORIGINAL ARTICLE

CHK2 stability is regulated by the E3 ubiquitin ligase SIAH2

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The serine threonine checkpoint kinase 2 (CHK2) is a critical protein involved in the DNA damage-response pathway, which is activated by phosphorylation inducing cellular response such as DNA repair, cell-cycle regulation or apoptosis. Although CHK2 activation mechanisms have been amply described, very little is known about degradation control processes. In the present study, we identify the ubiquitin E3 ligase SIAH2 as an interaction partner of CHK2, which mediates its ubiquitination and proteasomal degradation. CHK2 degradation is independent of both its activation and its kinase activity, but also of the phosphorylation in S456. We show that SIAH2-deficient cells present CHK2 accumulation together with lower ubiquitination levels. Accordingly, SIAH2 depletion by siRNA increases CHK2 levels. In response to DNA damage induced by etoposide, interaction between both proteins is disrupted, thus avoiding CHK2 degradation and promoting its stabilization. We also found that CHK2 phosphorylates SIAH2 at three residues (Thr26, Ser28 and Thr119), modifying its ability to regulate certain substrates. Cellular arrest in the G2/M phase induced by DNA damage is reverted by SIAH2 expression through the control of CHK2 levels. We observed that hypoxia decreases CHK2 levels in parallel to SIAH2 induction. Similarly, we provide evidence suggesting that resistance to apoptosis induced by genotoxic agents in cells subjected to hypoxia could be partly explained by the mutual regulation between both proteins. These results indicate that SIAH2 regulates CHK2 basal turnover, with important consequences on cell-cycle control and on the ability of hypoxia to alter the DNA damage-response pathway in cancer cells.

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INTRODUCTION

The serine threonine kinase CHK2 (checkpoint kinase 2) is a highly conserved protein critical in the DNA damage-response (DDR) signaling pathway.[1,2] Human CHK2 presents three different domains: an N-terminal region called SQ/TQ cluster domain (SCD), a forkhead-associated (FHA) domain and a canonical kinase domain in the C-terminal.[3–5] In response to DNA damage, mainly to double-strand breaks, CHK2 is phosphorylated in threonine 68 by ataxia telangiectasia mutated, which induces its dimerization and activation.[2,6,7]

CHK2 regulates several proteins involved in different steps of the DDR, such as DNA repair, cell-cycle regulation, apoptosis and p53 signaling.[8] To date, 24 substrate proteins of this kinase have been described in human cells, among which BRCA1, CDC25C, HDMX, PML and E2F1 are included.[2,9–13] CHK2 presents the ability to regulate p53 activity, either directly through Ser20 phosphorylation,[14] or by indirectly phosphorylating other proteins.[9,15]

Although CHK2 activation control mechanisms have been widely described,[16,17] very little is known about its inactivation and degradation processes. Serine/threonine protein phosphatase 2A (PP2A),[18] protein phosphatase 1D (WIP1)[19] and serine/threonine protein phosphatase 1 (PP1)[20] have been characterized as responsible to inactivate CHK2. Likewise, during the last decade several evidences have suggested that ubiquitination is an important control process in CHK2 turnover.[21] Even though two recent studies have identified E3 ubiquitin ligase RING finger protein 8 (RNF8) in humans[22] and p53-induced RING-H2 protein (PIRH2) in mice[23] as regulators of CHK2 through ubiquitination, the mechanisms required to maintain the steady levels remain poorly understood.

The E3 ubiquitin ligase SIAH2 (seven in absentia homolog 2) belongs to the RING (Really Interesting New Gene) finger E3 ubiquitin ligases.[24,25] In humans, two subunits (SIAH1 and SIAH2) have been described, which present similar and redundant functions.[26,27] SIAH2 is responsible for ubiquitination and proteosomal degradation of specific substrates, either through direct interaction or through binding to adapter proteins.[28–31] SIAH2 controls the expression of substrates involved in important signaling pathways like PML, HDAC3, TRAF2, β-catenin, HIPK2 and DYRK2.[28,30,32–34] However, its role on the regulation of hypoxia response stands out, mainly due to its ability to alter the expression of hypoxia inducible transcription factor 1-alpha (HIF-1α), which occurs through the degradation of the HIF-1α prolyl hydroxylases (PHDs).[35–37]

SIAH2 expression can be controlled at a transcriptional level by estrogens, different transcription factors (WNT5a and E2F1) and small noncoding microRNA (MiR-146b).[38–42] Different post-translational modifications able to modify SIAH2 activity have been described, such as their ability to autoubiquitinate and the phosphorylation mediated by upstream kinases.[43] To date, only three kinases able to phosphorylate SIAH2, modifying its activity, have been described: p38 MAPK,[44] and two members of the dual-specificity tyrosine-regulated kinase (DYRK) family, HIPK2 and DYRK2.[28,34]

Here we describe that SIAH2 interacts with CHK2 and mediates its ubiquitination and proteasomal degradation. Both proteins colocalize at the nucleus level and regulate mutually, and SIAH2 deficiency results in higher CHK2 levels.

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In response to DNA damage, interaction between both proteins is disrupted, thus promoting CHK2 stabilization. Moreover, we also prove the ability of CHK2 to phosphorylate SIAH2 in at least three residues, modifying its activity on certain substrates. In summary, we show the ability of SIAH2 to regulate CHK2 by ubiquitination, which represents a novel regulation mechanism of the expression and function for this relevant kinase.

RESULTS

SIAH2 interacts and colocalizes with CHK2

In order to investigate the mechanisms responsible for maintaining the steady levels of CHK2, we tested a set of different E3 ligases for their ability to interact with CHK2 (data not shown). As SIAH2 showed positive results in our screening, we decided to focus on this protein in detail. We first coexpressed Flag-CHK2 alone or in the presence of hemagglutinin epitope (HA)-tagged SIAH2 in HEK-293T cells, and performed coimmunoprecipitation assays. Flag-CHK2 coimmunoprecipitated efficiently with HA-SIAH2 (Figure 1a). Similar results were obtained the other way round (Figure 1b). Similarly, immunoprecipitation of endogenous CHK2 pulled down SIAH2 (Figure 1c).

Then, we analyzed the subcellular localization of both proteins and the effect of DNA damage. CHK2 and SIAH2 proteins mainly colocalize in the nucleus and no changes were observed in the cells stimulated with ETP (Figure 1d). Coimmunoprecipitation experiments showed that the interaction between these two proteins was markedly reduced under ETP (Figure 1e) and cisplatin stimulation and, to a lesser extent in response to 5-fluorouracil (Supplementary Figure S1). All these data support the existence of a CHK2-SIAH2 complex, which can be partially disrupted in response to DNA damage.

CHK2 shows a direct interaction with SIAH2

To study whether CHK2 and SIAH2 directly interact and to characterize the responsible domains, we performed GST-pulldown assays. Flag-CHK2 was brought down by full-length SIAH2 and also by two deletion mutants (Figure 2a). Similarly, Flag-SIAH2 was captured by full-length CHK2 and, to a lesser extent, by FHA or SCD domain-deleted mutants (Figure 2b). To identify the sites of direct interaction, we performed a peptide array experiment (Figure 2c). Detection of the bound material by antibodies showed that CHK2 binds four distinct domains of SIAH2. Likewise, SIAH2 showed interactions with two distinct sites of CHK2 (Supplementary Figure S2A). Then, we compared the interaction between SIAH2 and full-length CHK2 with FHA or SCD domain-deleted mutants. As shown in Supplementary Figure S2B, and in agreement with the results obtained in the GST-pulldown assays, FHA or SCD domain-deleted mutants were captured to a lesser degree than full-length CHK2 by Flag-SIAH2. Lastly, we decided to compare the interaction between SIAH2 and CHK2 wild type (wt) or CHK2 kinase dead mutant. These experiments revealed that the loss of CHK2 kinase activity does not affect the ability to interact with SIAH2 (Figure 2d). All these results clearly show a direct physical interaction of CHK2 and SIAH2, suggesting the existence of more than one domain responsible for binding in both proteins. Similarly, these data indicate that SIAH2 interacts with CHK2 monomeric and dimeric forms.

SIAH2 mediates CHK2 ubiquitination and proteosomal degradation

Based on the ability of SIAH2 to lead the ubiquitination and proteosomal degradation of many of its interaction partners, we analyzed whether SIAH2 can degrade CHK2 through an ubiquitin/proteasome-dependent process. We coexpressed CHK2 with increasing amounts of SIAH2 or the ligase-deficient SIAH2 point mutant (SIAH2 RM). Expression of SIAH2 resulted in a dose-dependent decrease in CHK2 protein levels in HEK-293T cells (Figure 3a). Similar results were obtained in MOR and A549 cells (Supplementary Figure S3A). By contrast, CHK2 levels were not altered in the presence of the SIAH2 RM. A comparative assay with SIAH1 showed that SIAH2 is the only member of the human family able to induce CHK2 degradation (Supplementary Figure S3B). Then, we examined this effect in the presence or absence of the proteasome inhibitor MG-132. The addition of this inhibitor stabilized SIAH2 and significantly prevented CHK2 degradation (Figure 3b).

Next, we decided to analyze the impact of SIAH2 expression at different levels on the endogenous CHK2. Increasing amounts of SIAH2 revealed a dose-dependent decrease in CHK2 protein levels without affecting mRNA expression (Figure 3c). Then we analyzed the effect of SIAH2 inhibition by siRNA and, based on the previous results by which DNA damage partially disrupted SIAH2–CHK2 interaction, also the effect of ETP stimulation. SIAH2 depletion increases CHK2 levels (Figure 3d) as well as its half-life (Supplementary Figure S4A), indicating that CHK2 basal levels can be regulated by this E3 ligase. In addition, ETP stimulation increased CHK2 stability as well as its half-life (Supplementary Figure S4A), which were not affected by SIAH2 inhibition. These changes were accompanied by an increase in CDC25C-Ser216 phosphorylation. Taken together, these results suggest that SIAH2 regulates CHK2 turnover through a mechanism occurring at the protein level, affecting its stability and activity.

We next examined the effect of SIAH2 on CHK2 ubiquitination *in vivo* and *in vitro*. We coexpressed His-Ubiquitin and HA-CHK2 with or without different concentrations of SIAH2 in the presence of MG-132, and analyzed the ubiquitination status of CHK2. As shown in Figure 3e, CHK2 polyubiquitination became more evident in the presence of increasing concentrations of SIAH2. By contrast, CHK2 polyubiquitination was inhibited in the presence of the SIAH2 RM (Supplementary Figure S3C). Then, we examined the effect of SIAH2 deficiency on the basal level of CHK2 polyubiquitination, comparing control mouse embryonic fibroblasts (MEFs) to Siah1a$^{-/-}$/Siah2$^{-/-}$ MEF cells. Ubiquitination levels of CHK2 were significantly lower in the knockout MEFs lacking Siah1a/2 (Figure 3f). Finally, we analyzed SIAH2 ability to directly ubiquitylate CHK2 through an *in vitro* ubiquitination assay. Polyubiquitination of CHK2 was observed only in the presence of SIAH2 (Supplementary Figure S4B).

CHK2 phosphorylation is a relevant mechanism for the control of its activity and stability. After DNA damage, T68 phosphorylation leads to conformational changes that induce dimerization and full activation.[45,46] Similarly, S456 phosphorylation is critical for the stability control by ubiquitination.[23,47,48] We decided to evaluate the effect of SIAH2 on CHK2 wt compared with CHK2 S456A and T68A mutants. As shown in Supplementary Figures S3D and S3E, both mutants were degraded in a similar way to CHK2 wt, indicating that the phosphorylation status of these residues is not relevant for the degradation mediated by SIAH2. Moreover, we compared the interaction between SIAH2 and CHK2 wt in the presence or absence of ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) inhibitor caffeine, or CHK2 S19A and T68A mutants. Coimmunoprecipitation experiments revealed that CHK2 phosphorylation status, at least in these important residues, is not relevant for the association with SIAH2 (Supplementary Figure S3F). In the same sense, we evaluated the capacity of SIAH2 to degrade FHA or SCD domain-deleted mutants (Supplementary Figure S3G). Although FHA domain-deleted mutant was partially degraded, the expression of the SCD domain-deleted mutant was not affected. Finally, we decided to study the effect of DNA damage on CHK2

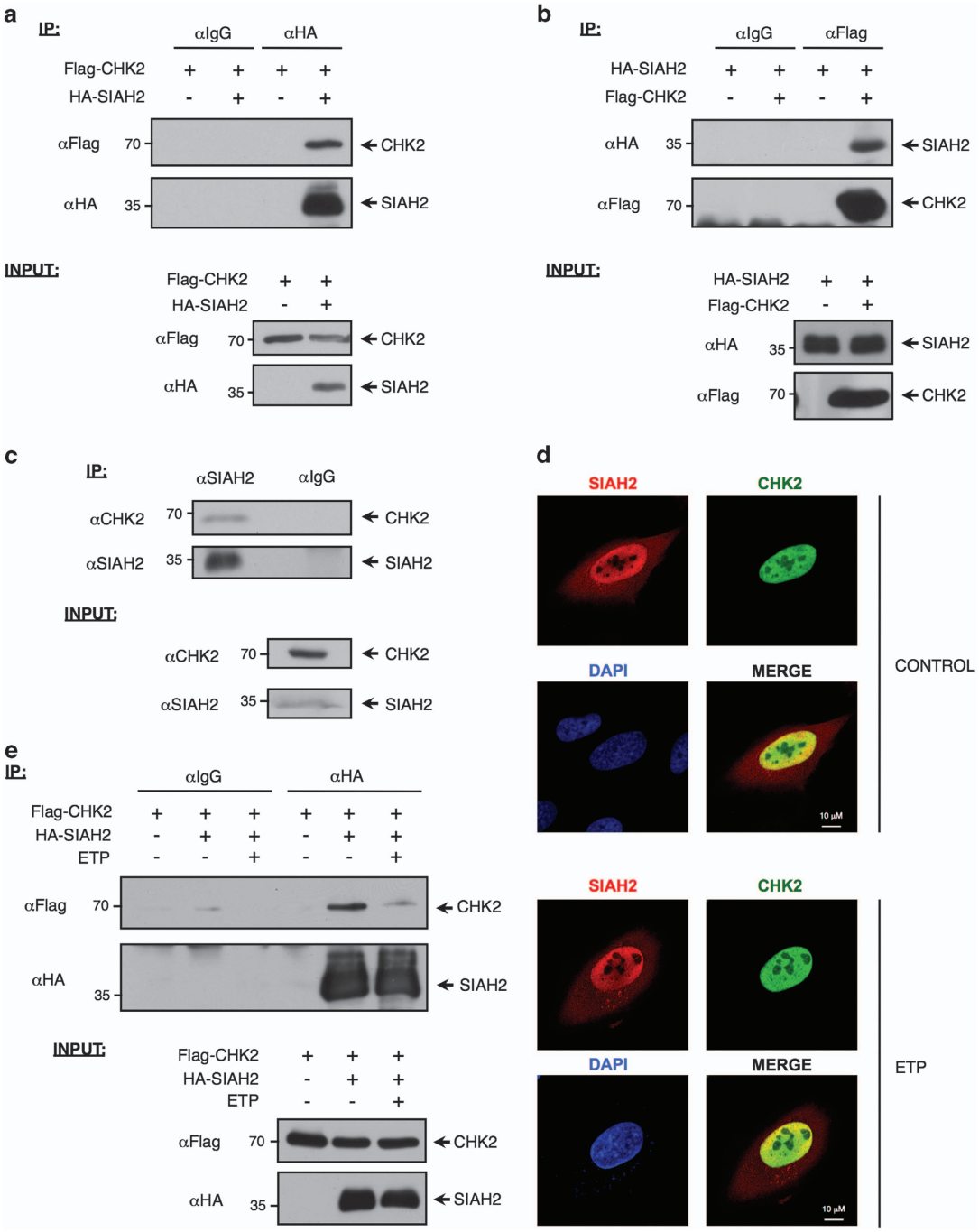


Figure 1. CHK2 interacts and colocalizes with SIAH2. (**a**) HEK-293T cells were transfected with expression plasmids encoding HA-tagged SIAH2 and Flag-CHK2 as indicated, and after 36 h the proteasome inhibitor MG-132 (10 μM) was added for another 12 h to avoid SIAH2 autodegradation. Cells were lysed and subjected to immunoprecipitation (IP) using anti-HA antibody. After elution, Flag-CHK2 protein was detected by western blotting. A small fraction (5%) of the lysate was tested for the occurrence of the indicated proteins by immunoblot (INPUT). The positions and molecular weights (in kDa) are indicated. We show a representative blot of three independent experiments. (**b**) The experimental settings were similar to (**a**) with the exception that the inmunoprecipitation was performed using anti-Flag antibody for CHK2. We show a representative blot of three independent experiments. (**c**) HeLa cells were lysed and a fraction subjected to IP with anti-SIAH2 antibody or Ig control. The precipitates were subjected to western blot analysis with anti-CHK2 or anti-SIAH2 antibodies. We show a representative blot of three independent experiments. (**d**) U2OS cells were transfected with Flag-SIAH2 RM (RING Mutant) and CHK2-GFP and analyzed for the localization of both proteins by immunofluorescence. Nuclear DNA was stained with DAPI. Overlapping localization in merged pictures is shown in yellow. We show a representative picture. Correlation analysis revealed a high degree of colocalization with a value of 0.59 ± 0.06 for Pearson's coefficient. No significant changes were observed in the cells stimulated with ETP (0.61 ± 0.07). (**e**) HEK-293T cells were transfected with the indicated plasmids, and after 36 h stimulated with etoposide (ETP) 10 μM as indicated and MG-132 for another 12 h. Cells were lysed, subjected to immunoprecipitation using anti-HA antibody, and the different proteins detected by western blotting. A small fraction (5%) of the lysate was tested by immunoblot for the occurrence of the indicated proteins (INPUT). We show a representative blot of four independent experiments.

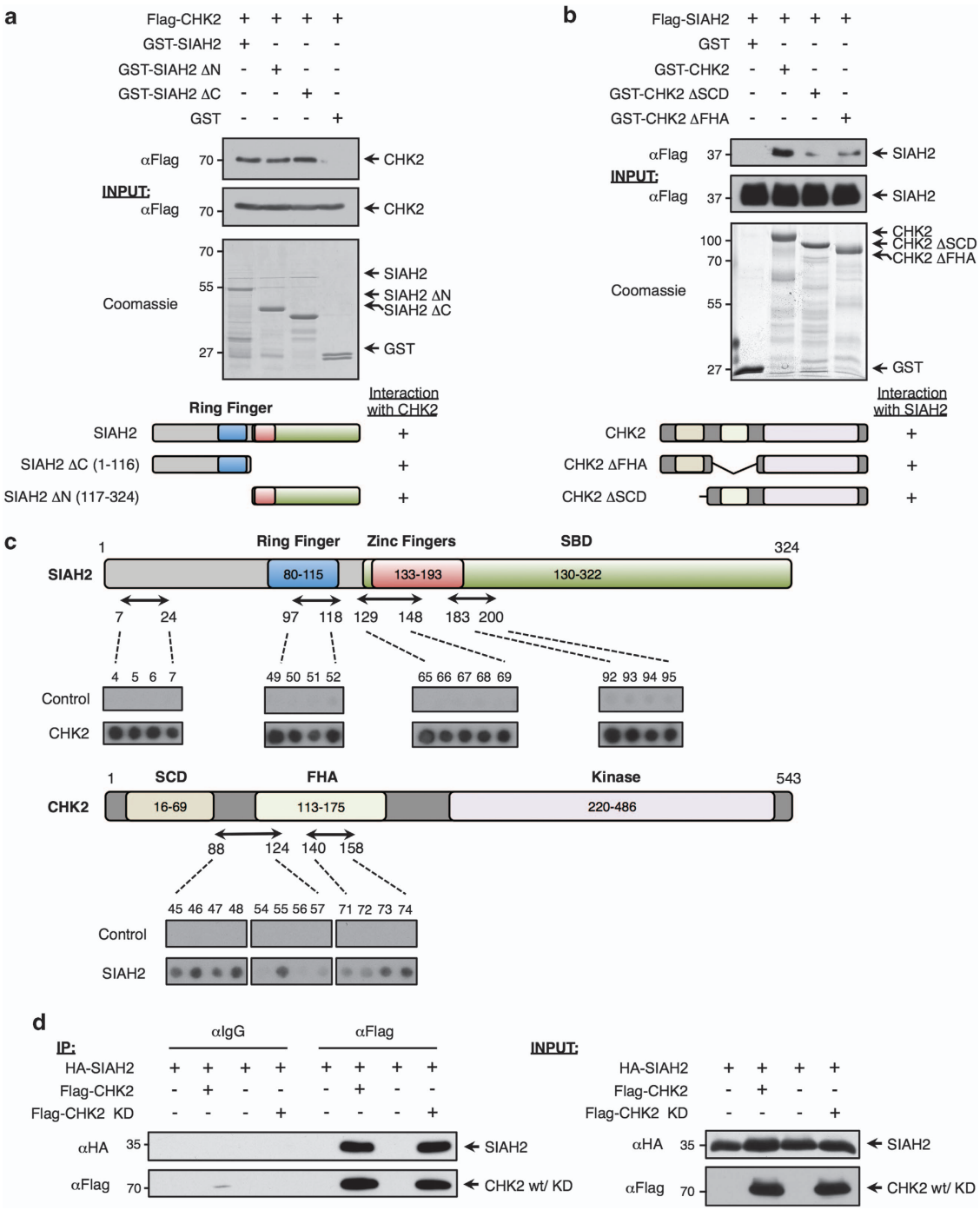


Figure 2. CHK2 shows direct interaction with SIAH2. (**a**) HEK-293T cells were transfected to express Flag-CHK2, lysed and incubated with GST-SIAH2 or deleted versions. After immunoprecipitation, CHK2 protein was detected by western blotting with anti-Flag antibody. The lower part shows the Coomassie-stained input material and a schematic representation of SIAH2-WT and its mutants. We show a representative blot of three independent experiments. (**b**) The experimental settings were similar to (**a**) with the exception that HEK-293T cells were transfected to express Flag-SIAH2, lysed and incubated with GST-CHK2 or deleted versions. We show a representative blot of three independent experiments. (**c**) Schematic representations of SIAH2 and CHK2 with the regions responsible for interaction. Recombinant GST-CHK2 or GST-SIAH2 proteins (or GST control protein) were incubated with a peptide array library covering the complete sequence of SIAH2 or CHK2, respectively, and bound proteins were revealed by immunoblot. CHK2 binds to four distinct domains of SIAH2, while SIAH2 interacts with two domains in the lower part of CHK2. Sequences of peptides interacting with CHK2 and SIAH2 are shown in Supplementary Figure S2A. We show a representative blot of two independent experiments. (**d**) HEK-293T cells were transfected with expression plasmids indicated, stimulated with MG-132 during 12 h, lysed and subjected to immunoprecipitation (IP) using anti-Flag antibody. Protein expression was detected by western blotting with the indicated antibodies. A fraction of the lysate was used to test the expression of the indicated proteins (INPUT). We show a representative blot of three independent experiments.

degradation by SIAH2. ETP stimulation prevented CHK2 polyubiquitination (Supplementary Figure S4C), degradation by SIAH2 (Figure 3g) and increased its half-life (Supplementary Figure S4A). Similar results were obtained after stimulation with cisplatin and 5-fluorouracil (Supplementary Figure S4D). Collectively, these data show that SIAH2 stimulates basal CHK2 polyubiquitination, thereby identifying CHK2 as a substrate for SIAH2-mediated ubiquitination and proteasomal degradation.

CHK2 phosphorylates SIAH2 in at least three residues *in vivo* and *in vitro*

As we showed in previous works,[28,34] some kinase substrates of SIAH2 have the ability to phosphorylate this E3 ligase. Therefore, we tested the capacity of CHK2 to phosphorylate SIAH2. CHK2 overexpression resulted in the presence of slower migrating SIAH2 bands, an effect not observed with CHK2 kinase dead mutant (KD) (Figure 4a). SIAH2 phosphorylation was analyzed with a specific phospho-SIAH2 antibody for Ser28.[28] To confirm that the upshifted SIAH2 bands were phosphorylated forms, we incubated cell extracts with λ-phosphatase. Treatment transformed the slower electrophoretic mobility bands into the faster migrating bands, and SIAH2 phosphorylation disappeared (Figure 4b). These results indicate that the upshifted SIAH2 bands observed after CHK2 wild-type overexpression represent hyperphosphorylated forms.

To examine the capacity of CHK2 to directly phosphorylate SIAH2, we performed an *in vitro* kinase assay. The presence of GST-CHK2 showed the occurrence of an upper band of GST-SIAH2 detected with a specific phospho-SIAH2 antibody (Figure 4c), which disappeared after λ-phosphatase treatment (Supplementary Figure S5). Next, we evaluated the CHK2 ability to phosphorylate endogenous SIAH2 by comparing phospho-SIAH2 endogenous levels of control cells to the cells where endogenous CHK2 was knock down by siRNA (Figure 4d). CHK2 inhibition caused a significant reduction of endogenous SIAH2-Ser28 phosphorylation. Taken together, these results clearly indicate that CHK2 can directly phosphorylate SIAH2.

To identify the SIAH2 sites phosphorylated by CHK2, we used four different phospho-specific antibodies (Thr26, Ser28, Ser68 and Thr119) previously described.[34] SIAH2 or point mutants of the phosphorylated amino acids to alanine were expressed either alone or together with CHK2, followed by detection of single-site phosphorylation (Figure 4e). CHK2 expression caused phosphorylation in three different sites (Thr26, Ser28 and Ser68), but we did not observe any significant changes in Thr119. Ser28 mutation precluded phosphorylation at Thr26, probably due to the fact that these sites are very close and in the same epitope. Once again, we examined the effect of ETP stimulation on SIAH2 phosphorylation by CHK2. As shown in Figure 4f and coherent with the previous results, stimulation with ETP decreased substantially SIAH2 phosphorylation levels mediated by CHK2. Overall, these results clearly indicate that CHK2 directly phosphorylates SIAH2 at a minimum of three residues.

Effect of SIAH2 phosphorylation on CHK2 turnover via ubiquitination and proteosomal degradation

Does SIAH2 phosphorylation by CHK2 control the degradation of this kinase through the ubiquitin/proteasome system? To address this issue, we transfected HEK-293T cells with Flag-CHK2 or Flag-CHK2 KD with increasing amounts of Flag-SIAH2. CHK2 degradation by SIAH2 was independent of the presence of its kinase activity (Figure 5a). Next, we compared the capacity of the phosphorylation (SIAH2-3A) and the phosphomimic (SIAH2-3D) mutants in the residues phosphorylated by CHK2 to degrade this kinase. Cells were transfected with Flag-CHK2 together with Flag-SIAH2-WT, Flag-SIAH2-3A or Flag-SIAH2-3D, using Flag-SIAH2 RM as a negative control. As shown in Figure 5b, CHK2 was degraded to a similar way in the three cases. These results support the hypothesis that the phosphorylation state of SIAH2 does not alter its capacity to degrade CHK2.

Others and we have revealed how SIAH2 phosphorylation affects its activity on certain substrates. Although the former results indicated that this is not the case for CHK2, we decided to analyze the effect on other substrates as prolyl hydroxylase 3 (PHD3). SIAH2 regulates hypoxia-inducible factor 1-alpha (HIF-1α) expression mostly via PHD3 inhibition.[35] Cells were transfected with HA-PHD3, together with different SIAH2 variants in the presence or absence of Flag-CHK2, and a luciferase construct controlled by the HIF-1-dependent erythropoietin promoter (Epo-Luc). SIAH2 produced an important reduction in PHD3 expression, which was reflected in a significant induction of HIF-dependent Epo-Luc promoter activity. SIAH2-3A mutant showed a reduced capacity to degrade PHD3, reflected in a lower response to the Epo-Luc promoter. On the contrary, the phosphomimetic SIAH2-3D mutant showed a strongly increased ability to degrade PHD3 and increase the Epo-Luc promoter response (Figure 5c). Coexpression of CHK2 strongly diminished PHD3 inhibition by SIAH2 and showed a significant reduction in the Epo-Luc promoter response. This effect, compared with the result obtained in response to the phosphomimetic SIAH2-3D mutant, suggests the possible CHK2 ability to phosphorylate SIAH2 in more residues with different consequences on its activity. Altogether, these experiments demonstrate that the SIAH2 phosphorylation mediated by CHK2 can regulate its capacity to degrade PHD3.

We subsequently investigated the effects of SIAH2 phosphorylation state in response to CHK2 on its capacity to degrade other two relevant SIAH2 substrates as DYRK2 and HIPK2. HEK-293T cells were transfected with kinase inactive mutants Flag-DYRK2 KD (Figure 5d) or Flag-HIPK2 KD (Supplementary Figure S6A) in order not to interfere with SIAH2 phosphorylation state, together with SIAH2 in the presence or absence of full-length CHK2, FHA or SCD domain-deleted mutants. In agreement with the PHD3 results, the presence of CHK2 significantly reduced HIPK2 and DYRK2 degradation. By contrast, FHA or SCD domain-deleted mutants, which lack the ability to phosphorylate SIAH2 (Supplementary Figure S6B), did not change the levels of both kinases. Altogether these data suggest that SIAH2 phosphorylation mediated by CHK2 does not affect its capacity to degrade this kinase, but modulates its ability to regulate other substrates such as PHD3, DYRK2 and HIPK2.

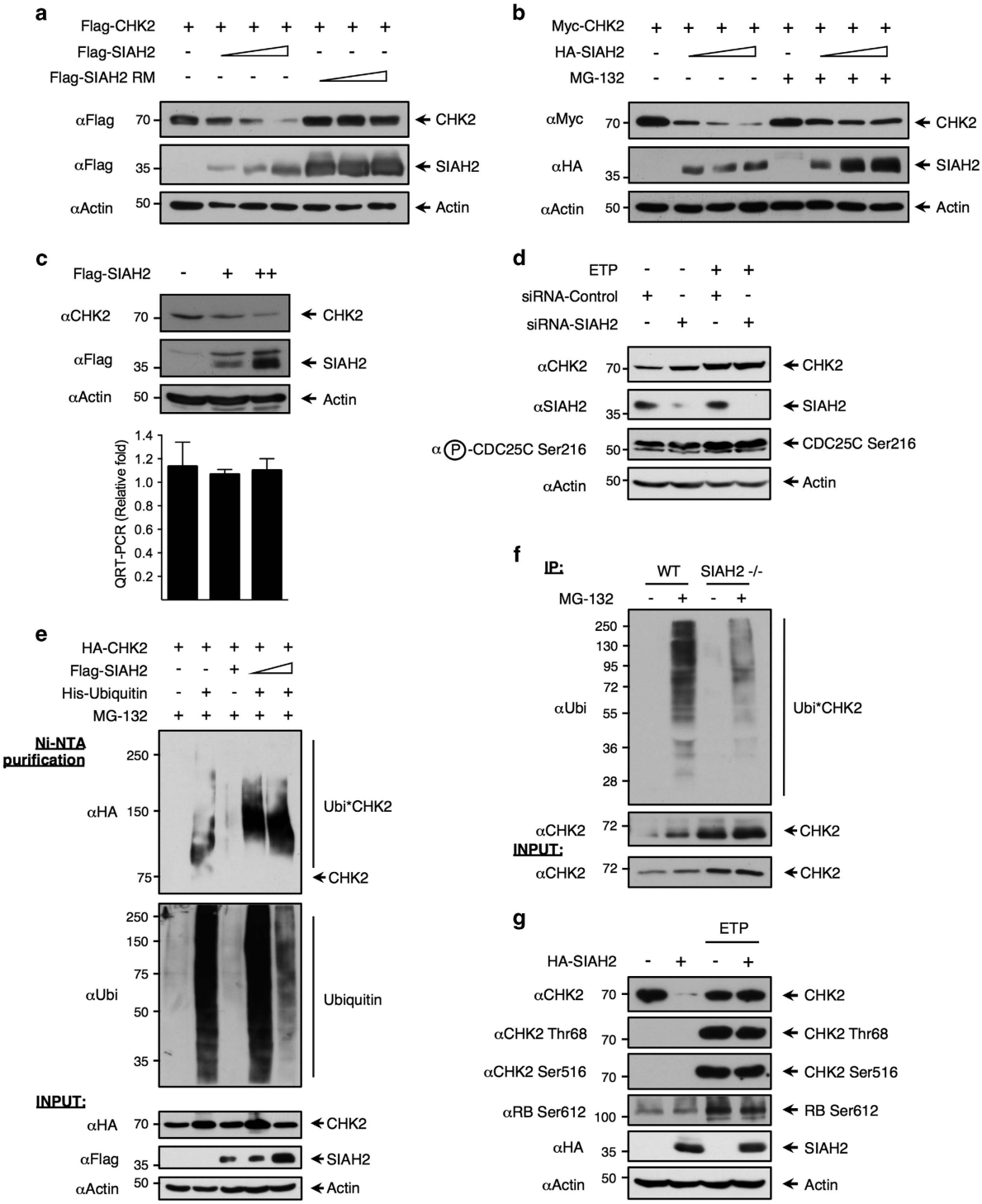
Effect of SIAH2 on CHK2 capacity to regulate cell-cycle arrest or apoptosis in response to DNA damage

CHK2 kinase has a relevant role in the DDR through cell-cycle checkpoint regulation.[2] Therefore, we tested the ability of SIAH2 to affect cell-cycle checkpoint activation in response to DNA damage. HEK-293T cells were transfected or not with SIAH2 and treated or not with ETP during 12 h. This time was taken to observe the direct effect on the cell-cycle arrest, which was analyzed together with the expression of CHK2, CDC25C and SIAH2 proteins. As depicted in Figure 6a, SIAH2 expression resulted in a clear reversion of the cellular arrest in the G2/M phase induced by the stimulation with ETP, presenting an inhibition of total and phosphorylated CHK2 (Thr68 and Ser516) and a reduction in phospho-CDC25C-Ser216 protein (CHK2 substrate).

We then investigated whether SIAH2 contributes to the apoptosis induced by DNA damage and the CHK2 role on this effect. HeLa cells were transfected with the indicated plasmids and stimulated with ETP. As shown in Figure 6b, the apoptosis percentage induced by stimulation with ETP was reduced in response to SIAH2 expression. In parallel, a significant reduction in the CHK2 expression was observed. Restoration of CHK2 protein

expression to its endogenous levels was associated with a significant recovery of apoptosis. Cell-cycle phase analysis was performed in parallel (Supplementary Figure S7A). In addition, we observed that SIAH2 expression increases cell viability in response to cytotoxic drugs (5-FU or Oxaliplatin) in HEK-293T (Figure 6c) and HeLa cells (Supplementary Figure S7B). Overall, these results indicate the SIAH2 ability to partially regulate the CHK2 capacity to control cell-cycle arrest or apoptosis in response to DNA damage.

Finally, we decided to study the functional consequences of CHK2-SIAH2 modulation in the context of hypoxia. SIAH2 is an essential component of the hypoxia response pathway with relevant implications on the carcinogenesis control.[35,49] Moreover, one of the causes of hypoxic tumor resistance to treatment is the hypoxia ability to alter the DDR signaling pathway.[50] To test this hypothesis, HeLa cells were subjected to hypoxia, stimulated with ETP and the apoptosis compared in control cells and cells where endogenous SIAH2 was knock down. Hypoxia reduced the



percentage of apoptosis in response to ETP, increasing SIAH2 levels and significantly reducing CHK2 expression. Moreover, SIAH2 depletion increased CHK2 levels, which was accompanied by a significant recovery of apoptosis (Figure 6d). Similar experiments were performed in A549 and MOR cells where, again, hypoxia produced a clear decrease in total and Thr68 CHK2 levels, thereby affecting its activity on CDC25C. SIAH2 knockdown under hypoxia conditions produced a significant increase in CHK2, which was also reflected in its activity (Supplementary Figures S8A and S8B). Similar results were obtained in the same cisplatin-resistant cell lines, thus indicating that this regulation is conserved in at least these two cell types. Collectively, all these results conclusively show that hypoxia regulates, at least partially, CHK2 stability and activity depending on SIAH2 expression levels. Similarly, they suggest that the resistance to apoptosis induced by genotoxic agents in cells subjected to hypoxia is due, at least in part, to the alteration of the DDR signaling pathway, which can be explained as a consequence of CHK2 depletion mediated by the expression of SIAH2.

DISCUSSION

The ubiquitin–proteasome pathway is critical for the expression and activity control of proteins implicated in cell-cycle control and DNA damage response.[51,52] CHK2 is a stable protein with a 6-h half-life whose regulation and degradation mechanisms in the absence of DNA damage are not completely known.[2,53] Although the phosphorylation levels and the action of several phosphatases are the most relevant mechanisms to keep CHK2 inactive,[18–20] different evidences suggest the role of ubiquitination to control its stability. Firstly, the capacity of Ub E3 ligase Mdm2 and acetyltransferase PCAF to increase CHK2 ubiquitination has been described, but in an independent way to their E3 ligase activities.[48] Similarly, the role of EDD in CHK2 activation has been reported, although its ability to mediate CHK2 ubiquitination has not been demonstrated yet.[54] Recently, the ability of the E3 ligase RNF8 in human, and PIRH2 in mice to ubiquitylate and degrade CHK2 has been characterized.[22,23] It should be underlined that, whereas these two E3 ligases mediate degradation in response to DNA damage, our results suggest that SIAH2 regulates CHK2 basal expression.

In the present work we describe the capacity of CHK2 to directly interact *in vivo* with SIAH2, mainly colocalizing in the nucleus. Through different experimental approaches, we prove that SIAH2 mediates CHK2 ubiquitination and proteosomal degradation. SIAH2 depletion increases CHK2 levels, thereby supporting our observation that SIAH2-deficient cells show high CHK2 levels. Phosphorylation in S456, which has been proved to be critical for CHK2 ubiquitination, is not here relevant for CHK2 degradation.[47] Similarly, our results show that SIAH2-mediated CHK2 degradation is independent of both phosphorylation in T68 and its kinase activity. Likewise, DNA damage alters the interaction between both proteins, thus decreasing SIAH2-mediated CHK2 levels and not affecting the endogenous levels of CHK2 T68 or S516. These results suggest that SIAH2 interacts and regulates a pool of CHK2 in the absence of stimuli. In response to DNA damage, a CHK2 fraction would split from SIAH2, thus avoiding its degradation and allowing its stabilization. Although the exact mechanism underlying the separation of SIAH2 from CHK2 after DNA damage is unknown, it seems reasonable to think that it could be the result of post-transductional modifications suffered by any of the two proteins in response to this stimulus. The absence of a complete dissociation of both proteins in response to DNA damage observed in our results could explain the existence of different CHK2 fractions controlled by SIAH2 contributing to the cell-cycle control (Figure 7).

The regulation of ubiquitin ligases activity is critical for the control of its substrates. Different control mechanisms have been described for SIAH family, among which the role of phosphorylation stands out.[42] Others and we have reported that SIAH2 phosphorylation affects its activity, and three kinases with SIAH2 as substrate have been described so far.[28,34,44] In this work we also highlight CHK2 capacity to phosphorylate SIAH2 in at least three residues, not affecting its activity on this kinase, but decreasing its ability to degrade other substrates. These results are consistent with previous reports describing how SIAH2 phosphorylation modulates its activity specifically on certain substrates.[34,55,56] Similarly, our results show how ETP stimulation significantly reduces CHK2-mediated SIAH2 phosphorylation, in agreement with the loss of interaction between these two proteins. As a consequence, the activity of SIAH2 on some of its substrates in response to DNA damage would be modified. The changes observed in some SIAH2 substrates in response to DNA damage such as DYRK2 are in accord with this hypothesis.[57,58]

From the functional point of view, CHK2 plays a relevant role in the cell-cycle control in response to DNA damage, inhibiting CDC25C phosphatase through Ser216 phosphorylation and avoiding mitosis.[2,59] Our results demonstrate how SIAH2 expression resulted in a clear reversion of cellular arrest induced by ETP stimulation. Besides, we demonstrate that the loss of SIAH2-mediated CHK2 partially prevented ETP-induced apoptosis on HeLa cells, which was recovered by returning CHK2 protein expression to endogenous levels. These data prove that SIAH2

Figure 3. SIAH2 mediates ubiquitin/proteasomal degradation of CHK2. (**a**) HEK-293T cells were transfected to express CHK2 and the levels were evaluated in response to increasing concentrations of SIAH2 wild type or SIAH2 RING mutant by western blotting. We show a representative blot of three independent experiments. (**b**) Cells were transfected with the indicated plasmids to express CHK2 and increasing concentrations of SIAH2 in the presence or absence of MG-132. Cells were lysed and protein expression analyzed by immunoblot. We show a representative blot of three independent experiments. (**c**) HEK-293T cells were transfected to express increasing amounts of SIAH2, harvested and lysed. One fraction was used to analyze the endogenous CHK2 protein levels while another aliquot was used to analyze the CHK2 mRNA levels by quantitative PCR. Data are mean ± s.d. of $n = 3$. We show a representative blot of three independent experiments. Primer sequences are available upon request. (**d**) HEK-293T cells were transfected with SIAH2 or scrambled (control) siRNAs, after 4 days stimulated with etoposide (10 μM) during 24 h as indicated, lysed and protein expression analyzed by immunoblot. We show a representative blot of three independent experiments. (**e**) HEK-293T cells were transfected with expression plasmids encoding HA-tagged CHK2, Flag-tagged SIAH2 and His-tagged ubiquitin. After 36 h cells were incubated in the presence of MG-132 (10 μM) during 12 h and lysed under denaturing conditions. His-tagged ubiquitin was purified with Ni-NTA agarose columns and ubiquitinated CHK2 was analyzed by western blotting. A fraction was tested for the occurrence of the indicated proteins (INPUT). We show a representative blot of three independent experiments. (**f**) Wild type and Siah1a$^{-/-}$/Siah2$^{-/-}$ MEF cells were stimulated or not with MG-132 during 12 h, lysed and subjected to immunoprecipitation using anti-CHK2 antibody. The precipitates were subjected to western blot analysis with anti-CHK2 or anti-Ub antibodies. A small fraction of the lysate was tested for the occurrence of CHK2 (INPUT). We show a representative blot of three independent experiments. (**g**) HEK-293T cells were transfected or not to express SIAH2, and 24 h post-transfection stimulated with etoposide (10 μM) during 24 h as indicated. Cells were lysed and protein expression was analyzed by immunoblot with the indicated antibodies. We show a representative blot of three independent experiments.

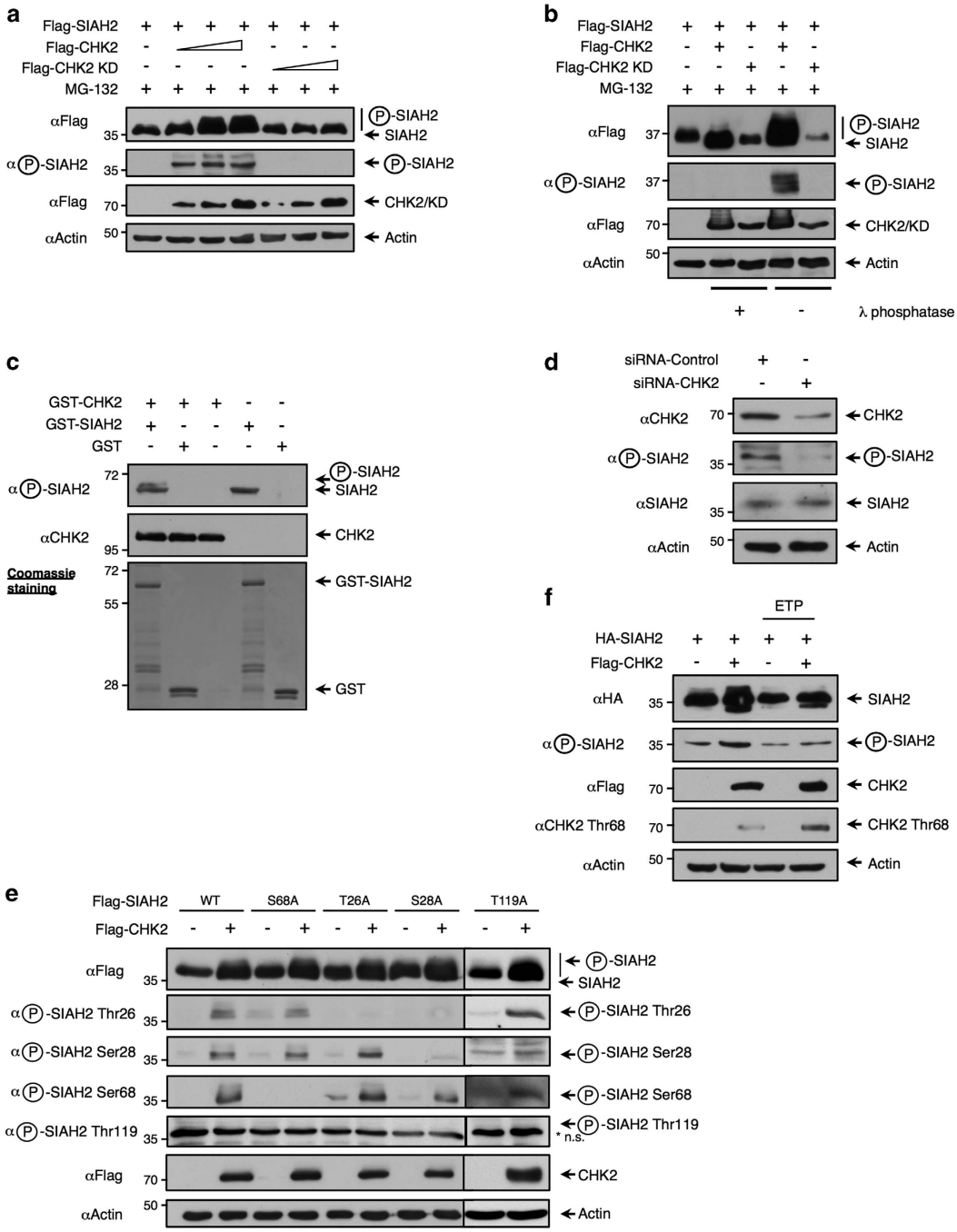


Figure 4. CHK2 phosphorylates SIAH2 *in vivo* and *in vitro*. (**a**) HEK-293T cells were transfected to express Flag-SIAH2 and increasing amounts of Flag-CHK2 wild type or Flag-CHK2 kinase dead mutant (KD), and after 36 h treated for 12 h with the proteasome inhibitor MG-132 (10 μM). Cell lysates were analyzed by immunoblotting with the indicated antibodies. SIAH2 phosphorylation was seen by the upshifted band and also by the use of a specific antibody recognizing phosphorylated Ser28 of SIAH2. We show a representative blot of three independent experiments. (**b**) HEK-293T cells were transfected with the indicated plasmids and treated with MG-132 for 12 h. Cells were lysed in phosphatase inhibitor-free buffer in the absence or presence of λ-phosphatase. Electrophoretic mobility was determined by immunoblotting and in parallel SIAH2 phosphorylation was analyzed with phospho-specific antibody for Ser28 of SIAH2. We show a representative blot of three independent experiments. (**c**) *In vitro* kinase assay was performed with active CHK2 recombinant protein and purified GST-SIAH2. Phosphorylation was determined with phospho-specific antibody for Ser28 of SIAH2, while protein levels were visualized by Coomassie staining (lower). (**d**) HEK-293T cells were transfected with CHK2 or scrambled (control) siRNAs, lysed after 4 days of culture and CHK2 or SIAH2 phosphorylation analyzed by western blot. We show a representative blot of three independent experiments. (**e**) HEK-293T cells were cotransfected with Flag-SIAH2 or the indicated SIAH2 point mutants either alone or along with CHK2 and treated with MG-132 for 12 h. Electrophoretic mobility was determined by western blotting and in parallel SIAH2 phosphorylation was analyzed with phospho-specific antibodies for the residues indicated. We show a representative blot of three independent experiments. (**f**) Cells were transfected to express HA-SIAH2 in the presence or absence of Flag-CHK2. Twenty-four hours post-transfection cells were stimulated with etoposide (6 μM) during 24 h, lysed and protein expression was analyzed by immunoblot with the indicated antibodies. SIAH2 phosphorylation was analyzed with phospho-specific antibody for Ser28. We show a representative blot of three independent experiments.

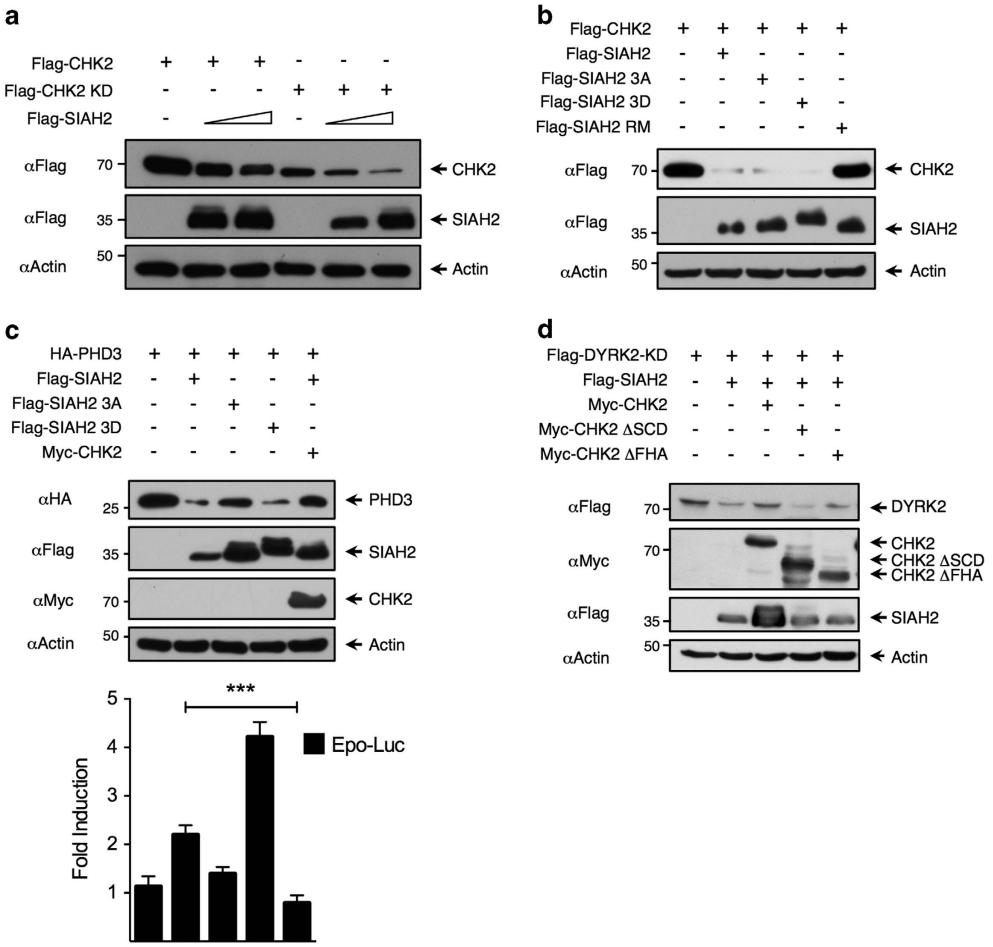


Figure 5. Regulation of SIAH2 activity by CHK2. (**a**) HEK-293T cells were transfected to express Flag-CHK2 or Flag-CHK2 KD (Kinase mutant) with increasing amounts of SIAH2, lysed and protein expression was analyzed by immunoblot with the indicated antibodies. We show a representative blot of four independent experiments. (**b**) Cells were transfected with Flag-CHK2 and the plasmids encoding SIAH2 wild type and the phosphorylation (SIAH2-3A) and the phosphomimic (SIAH2-3D) mutants in the residues Thr26, Ser28 and Ser68. After 36 h cells were lysed and the stability of CHK2 was revealed by immunoblotting. We show a representative blot of three independent experiments. (**c**) HEK-293T cells were transfected with the indicated plasmids together with a luciferase construct controlled by the HIF-1-dependent erythropoietin promoter (Epo-Luc). After 36 h, cells were lysed and further analyzed for the indicated proteins by immunoblots (upper panel) or for luciferase expression (lower panel). Data are mean ± s.d. of $n = 3$ experiments. ***$P < 0.001$. We show a representative blot of three independent experiments. (**d**) HEK-293T cells were transfected with the indicated plasmids, and after 36 h lysed and protein expression evaluated by immunoblot with the indicated antibodies. We show a representative blot of three independent experiments.

expression affects cell-cycle activation checkpoints through the control of CHK2 levels.

Finally, many studies support the concept that hypoxia is directly implicated in tumor progression and metastasis, mediating apoptosis resistance and reducing the ability to repair DNA.[50,60] Different works have reported how intratumoral hypoxia significantly modifies the action of some drugs and promotes metastasis.[60,61] Similarly, cells subjected to hypoxia present a lower capacity to repair DNA and, as a consequence, they become more resistant to chemotherapy.[62,63] Different clinical trials that attempted to diminish tumor hypoxia have significantly improved cell sensibility to radiotherapy and chemotherapy, which resulted in a higher rate of survival.[64] In general, the concept that hypoxia increases cell resistance to apoptosis through different mechanisms is consistent with our results. SIAH2 has the capacity to regulate HIF-1α levels, and thus the physiological response to hypoxia.[35] We observed that hypoxia decreases CHK2 levels, parallel to an increase of both SIAH2 and cell resistance to apoptosis induced by ETP. We have also observed how SIAH2 expression increases cell viability in response to cytotoxic drugs such as 5-FU or Oxaliplatin. These data are equally supported, at least partially, by previous results which also showed lower CHK2 levels under hypoxia in HeLa and MCF-7 cells.[65] Altogether, this finding suggests a new action mechanism by which hypoxia may alter the DDR pathway via SIAH2–CHK2 modulation.

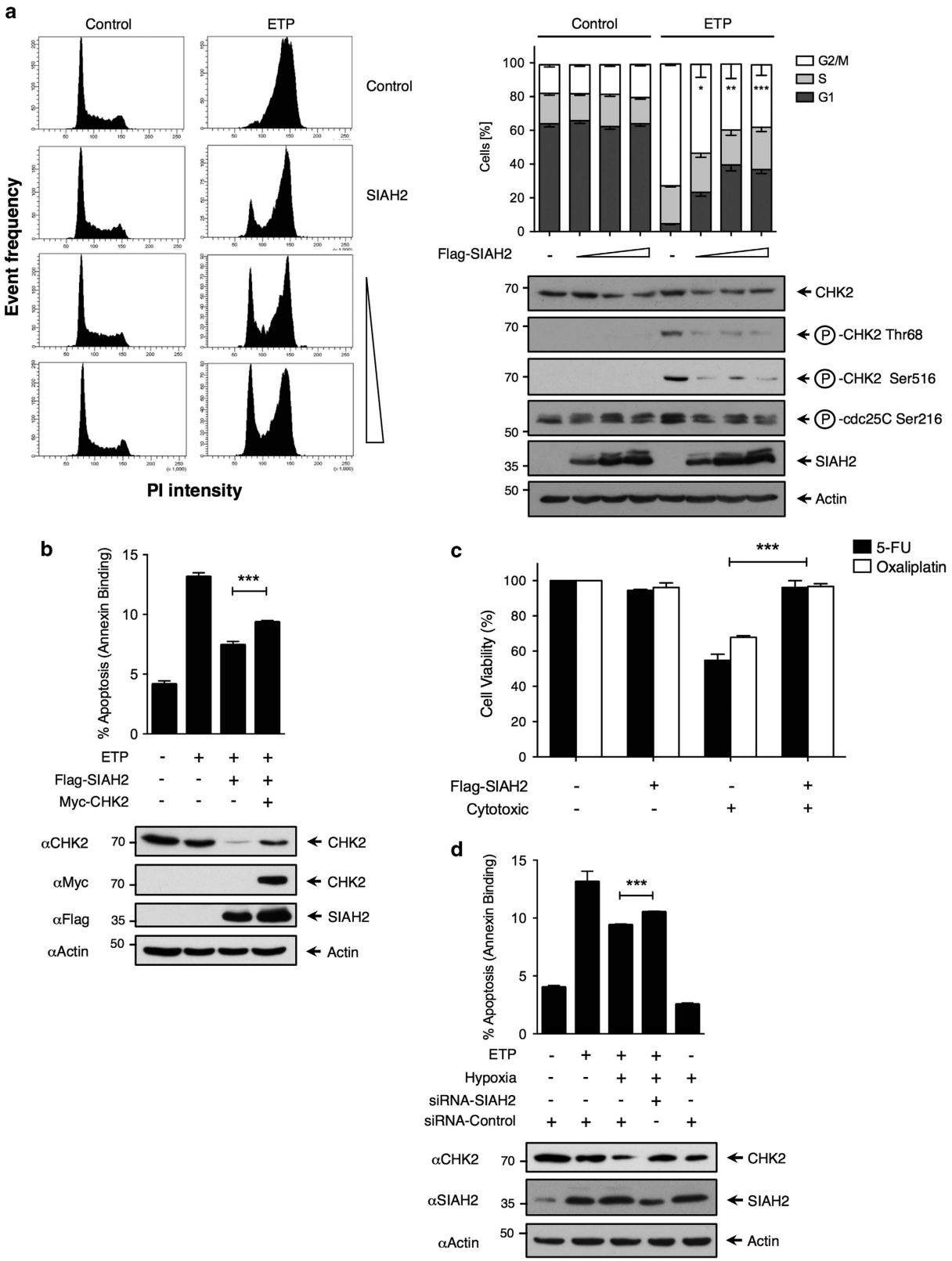
In summary, our results indicate that E3 ubiquitin ligase SIAH2 regulates CHK2 basal turnover. Additionally, CHK2 presents the ability to phosphorylate SIAH2, affecting its activity on certain substrates. In response to DNA damage, the interaction between both proteins is altered, which favors CHK2 stabilization. We propose that this new CHK2 regulation mechanism has an influence on cell-cycle control and on the ability of hypoxia to alter the DDR pathway in cancer cells. Further studies of the relationship of these two proteins in tumors are needed to support this hypothesis.

MATERIALS AND METHODS

Cell cultures and reagents

HEK-293T, HeLa, U2OS, A549 (wt/CPR), control MEFs and Siah1a$^{-/-}$/Siah2$^{-/-}$ MEF cells were maintained in Dulbecco's modified Eagle's medium, MOR (wt/CPR) in RPMI, both supplemented with 10% fetal calf serum,

2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cell lines are routinely tested to be free of mycoplasma and cross contamination. Cell lines validation was performed by a multiplex PCR with Geneprint10 System (Promega, Madison, WI, USA). HEK-293T, HeLa, U2OS and control MEFs cells were obtained from ATCC (LGC Standards, Teddington, Middlesex, UK). A549 wt/CPR cells and Epo-Luc plasmid were a gift from Dr M L Schmitz (University of Giessen, Germany). MOR wt/CPR were from Sigma-Aldrich (St Louis, MO,



USA). Siah1a$^{-/-}$/Siah2$^{-/-}$ MEF cells were obtained from Dr David Bowtell and Dr Andreas Möller. MG-132 was from Enzo Life Science (Lausen, Switzerland), and the rest of the reagents were from Sigma-Aldrich. Scramble control oligonucleotide siRNA non-targeting pool (D-001810-10-20) and the siGENOME SMARTpool against SIAH2 (M-006561-02) and ON-TARGET plus SMARTpool against CHK2 (L-003256-00) were purchased from Dharmacon (Waltham, MA, USA). E1, E2-UbcH5c and ubiquitin human recombinant proteins were purchased from Abcam (Cambrigde, UK).

Transfection and plasmids

Transient transfections, SIAH2 plasmids and His-Ubiquitin were previously described.[28,34] Flag-CHK2, KD and T68A, Myc-CHK2, KD were a gift from Dr H Piwnica-Worms (Washington University School of Medicine, St Louis, MO, USA). GST-CHK2 plasmid was a gift from Dr Stephen J Elledge (Harvard Medical School, Boston, MA, USA). HA-CHK2, KD, GST-CHK2-ΔSCD and ΔFHA plasmids were kindly provided by Dr David F Stern (Yale University School of Medicine, New Haven, CT, USA). HA-CHK2 S456A was provided by Dr Carol Prives (Department of Biological Sciences, Columbia University, New York, NY, USA). CHK2-GFP was provided by Dr L Zannini (Istituto Nazionale dei Tumori, Milan, Italy). Myc-CHK2 ΔSCD and ΔFHA were kindly provided by Dr S-Y Shieh (Institute of Biomedical Science, Taiwan). Flag-CHK2 S19A was a gift from Dr Domenico Delia (Istituto Nazionale dei Tumori, Milan, Italy). PHD3 plasmid was a gift from Dr Frank S Lee (Pennsylvania School of Medicine, Philadelphia, PA, USA).

Cell lysis, western blot, immunoprecipitation analysis, immunofluorescence and antibodies

Cells lysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotting, immunoprecipitation, enrichment of His-tagged proteins and Immunofluorescence were described by us previously.[28,34] Mouse monoclonal antibodies against Flag (M2) and anti-β-actin (AC-74) were purchased from Sigma-Aldrich, anti-HA epitope (3F10) and anti-myc (9E10) from Roche Molecular Biochemicals (Mannheim, Germany), anti-p53 phospho Ser46 (2521), anti-ubiquitin (P4D1), anti-CHK2 Thr68 [(C13C1)-2197], anti-CDC25C Ser216 (9528), anti-CHK2 Ser516 (2669) and anti-p53 Ser20 (9287) from Cell Signaling Technology (Danvers, MA, USA), anti-SIAH2 (sc-5507) and anti-p53 (sc-126) from Santa Cruz (Santa Cruz, CA, USA). Anti-phospho-SIAH2 antibodies were previously described.[28,34] Alexa Fluor 647 goat anti-mouse IgG1 (A21235) antibody was from Life technologies (Carlsbad, CA, USA). Secondary horseradish peroxidase -coupled antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Hypoxia, GST-pulldown experiments, *in vitro* ubiquitination and phosphorylation, peptide array-binding assays, quantitative reverse transcriptase–PCR, luciferase reporter and cell viability assays and flow cytometry analyses

Hypoxia, GST-pulldown experiments, *in vitro* phosphorylation, peptide array-binding assays and quantitative reverse transcriptase–PCR,[34] *in vitro* ubiquination and flow cytometry analyses,[66] Luciferase and cell viability assays[67] were performed as described previously.

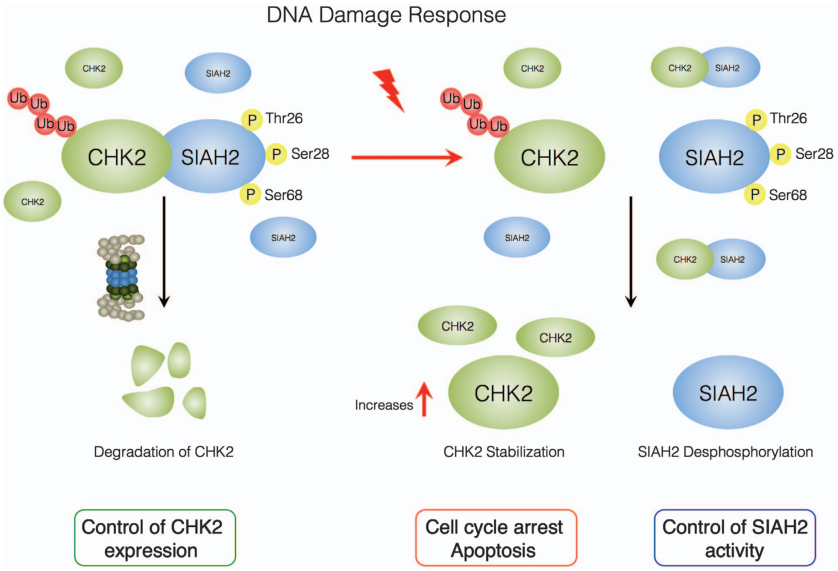


Figure 7. Schematic model for the ability of SIAH2 to regulate CHK2 by ubiquitination. Under normal conditions SIAH2 is able to interact and modulate the basal turnover of CHK2 by ubiquitination and proteasome degradation. Simultaneously, CHK2 is able to phosphorylate SIAH2 in at least three residues (Thr26, Ser28 and Ser68). The DNA damage response causes alteration in the protein–protein interaction, promoting CHK2 stabilization and SIAH2 activity modification.

Figure 6. Functional consequences of CHK2 regulation by SIAH2. (**a**) HEK-293T cells were transfected with increasing amounts of SIAH2 and after 12 h stimulated with etoposide (6 μM) during 12 h. A fraction of the cells was used for cell-cycle analysis (left panel) using flow cytometry. The left panel shows representative cell-cycle distributions. The right panel shows quantitation of percentages of the cells in each phase of the cell-cycle. Data are mean ± s.d. of $n = 3$ experiments. Another aliquot (lower panel) was lysed and protein expression was analyzed by immunoblot with the indicated antibodies. We show a representative blot of three independent experiments. (**b**) HeLa cells were transfected with the indicated plasmids and 24 h later stimulated with ETP (10 μM) for another 24 h. One aliquot was used for apoptosis analysis determined by flow cytometry using Annexin V/PI staining (upper panel), while another fraction was lysed and the levels of the indicated protein analyzed by immunoblot. Data are mean ± s.d. of $n = 3$ experiments. ***$P < 0.001$. (**c**) HEK-293T cells were transfected or not to express SIAH2 and 24 h later stimulated as indicated with 5-fluorouracil (5-FU, 0.5 μg/ml) or Oxaliplatin (0.5 μM) during 24 h. Cell viability was measured by MTT assay. Data are mean ± s.d. of $n = 3$ experiments. *$P = 0.01$; **$P = 0.002$; ***$P < 0.001$. (**d**) HeLa cells were transfected with the indicated siRNA and 24 h later stimulated with ETP 10 μM for another 24 h. Cells were subjected to hypoxia (1% O_2) during 12 h and one fraction used for apoptosis analysis by Annexin V/PI staining (upper panel), while another fraction was lysed and the levels of the indicated protein were analyzed by immunoblot. Data are mean ± s.d. of $n = 3$ experiments. ***$P < 0.001$.

Statistical analysis

Data are expressed as mean ± s.d.. Differences were analyzed by Student's *t*-test. Statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered significant. Images were analyzed and quantified using the ImageJ v1.45 software.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)